Unwanted hydrolysis or α/β-peptide bond formation? How long the rate-limiting coupling step should take?

Viktória Goldschmidt Gőz,[1] Adrienn Nagy,[2] Viktor Farkas,[1] Ernő Keszei,[3] and András Perczel*[1,2]

[1]*MTA-ELTE Protein Modeling Research Group, Pázmány P. sétány. 1/A, 1117 Budapest, Hungary*

[2]*Laboratory of Structural Chemistry and Biology, Institute of Chemistry, Eötvös Loránd University, Pázmány P. stny. 1/A, 1117 Budapest, Hungary*

[3]*Chemical Kinetics Laboratory, Institute of Chemistry, Eötvös Loránd University, Pázmány P. stny. 1/A, 1117 Budapest, Hungary*

* *e-mail:* perczel.andras@ttk.elte.hu

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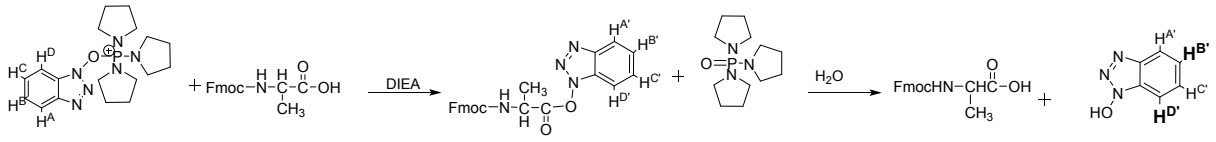
1. Active ester formation with HOBt/DIC

Table 1. Reaction times (min) and conversions (%) for the active ester formation (f) and hydrolysis (h) for Fmoc protected α- and β-amino acids with HOBt/DIC. Conversion after 10, 60 minutes and 3 hours are shown which is the limit of the couplings.

Active esters with HOBt/DIC	Formation (f)		Hydrolysis (h)		Conversion (%) of formation/hydrolysis		
	t_f (min)	conversion (%)	t_h (min)	conversion (%)	10 min	60 min	180 min
Fmoc-Val-OH	240	>99	∞	0	f: 22	f: 62	f: 95
Fmoc-Arg-OH	120	35	1440	100	f: 3	f: 17	f: 30
Fmoc-RibAFU(ip)-OH	60	55	1440	100	f: 28	f: 55	h: 46
Fmoc-GlcAPU(Me)-OH	120	73	∞	0	f: 21	f: 48	f: 73

2. ^{1}H-NMR Spectra and diagrams of the active esters formation and hydrolysis with PyBOP/DIEA

2.1. Fast hydrolyzing active esters



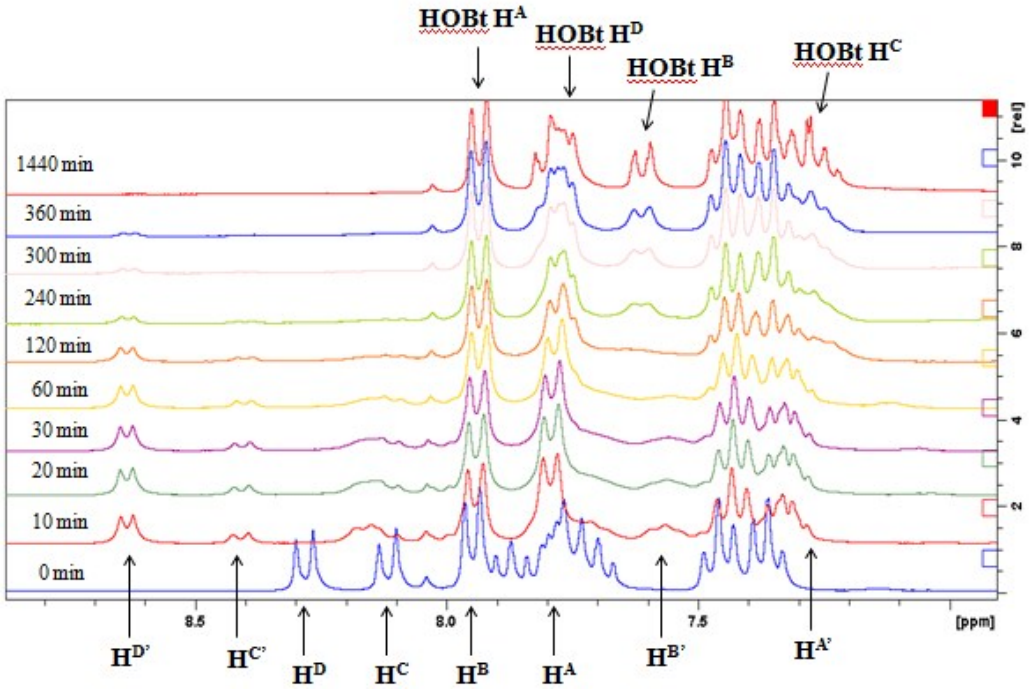


Figure 1. Reaction of Fmoc-Ala-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic range, 250 MHz

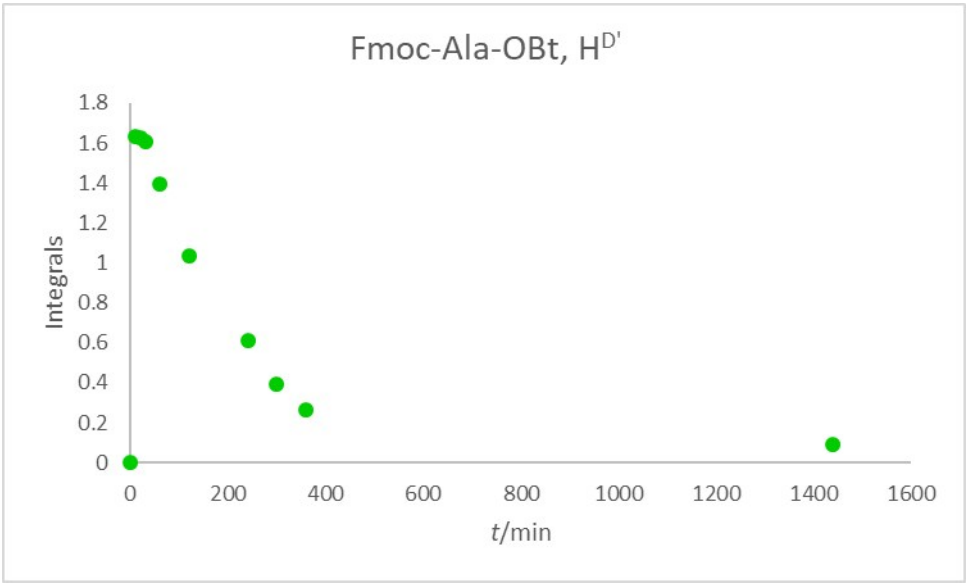


Figure 2. Integral-time diagram of Fmoc-Ala-OBt active ester, H^D' signal at 8.63 ppm

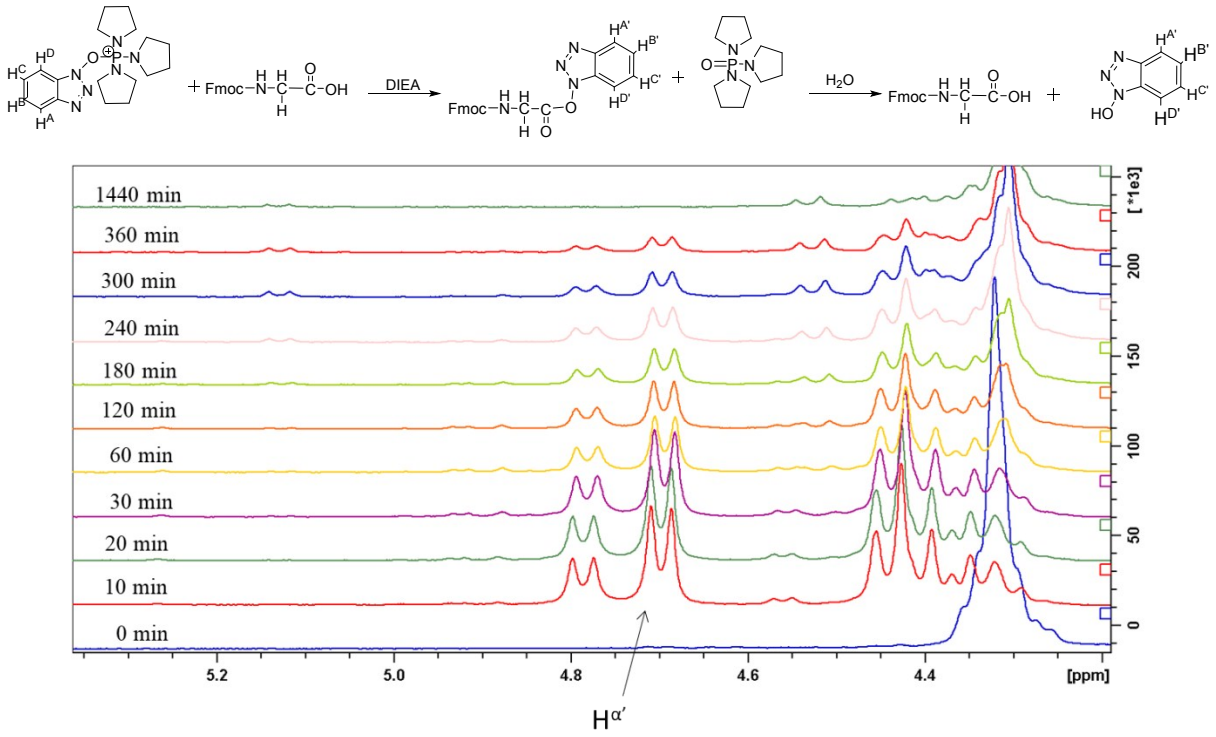


Figure 3. Reaction of Fmoc-Gly-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aliphatic region, 250 MHz

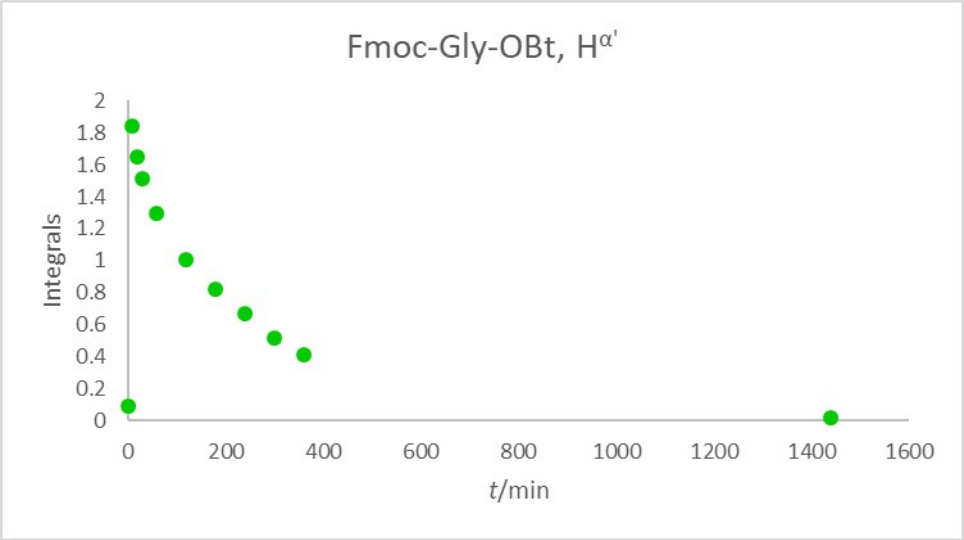


Figure 4. Integral-time diagram of Fmoc-Gly-OBt active ester, Hα' signal at 4.70 ppm

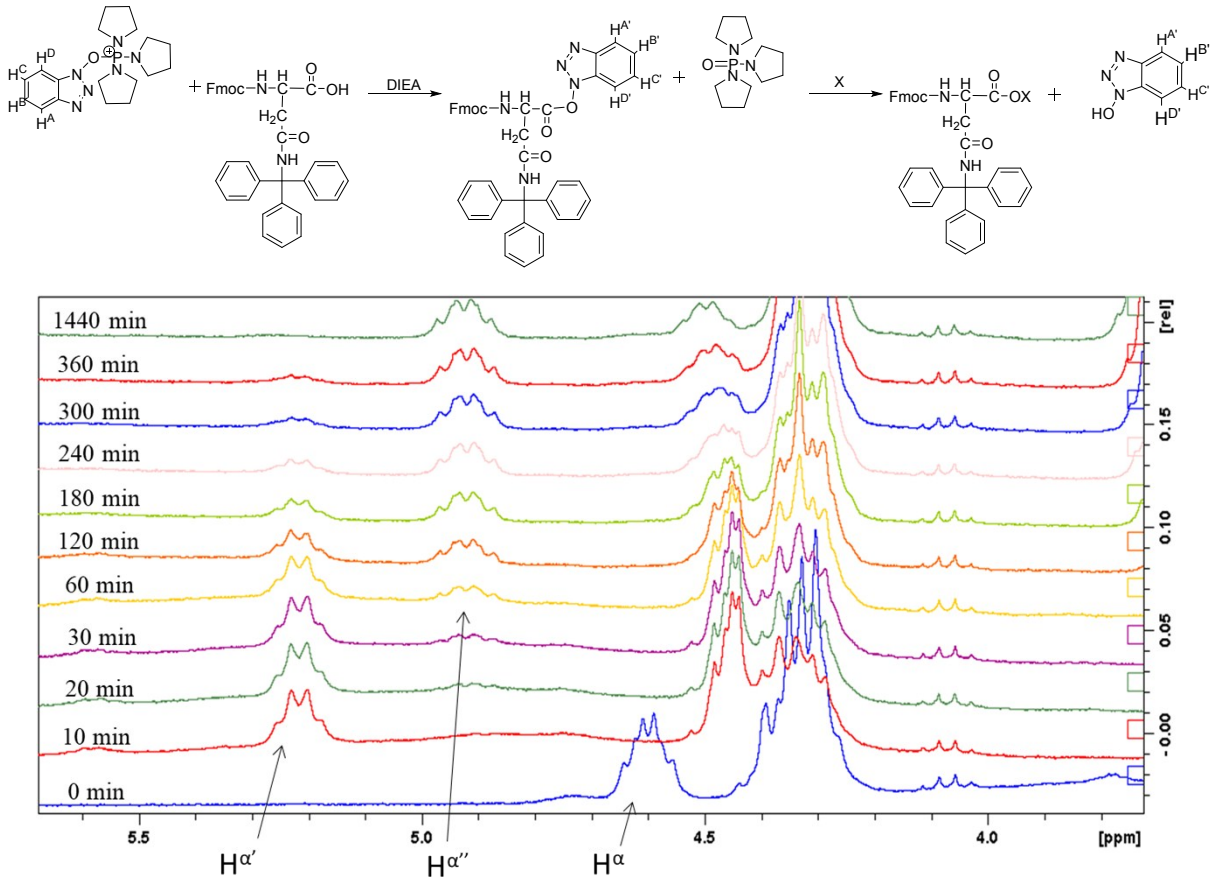


Figure 5. Reaction of Fmoc-Asn(Trt)-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aliphatic region, 250 MHz

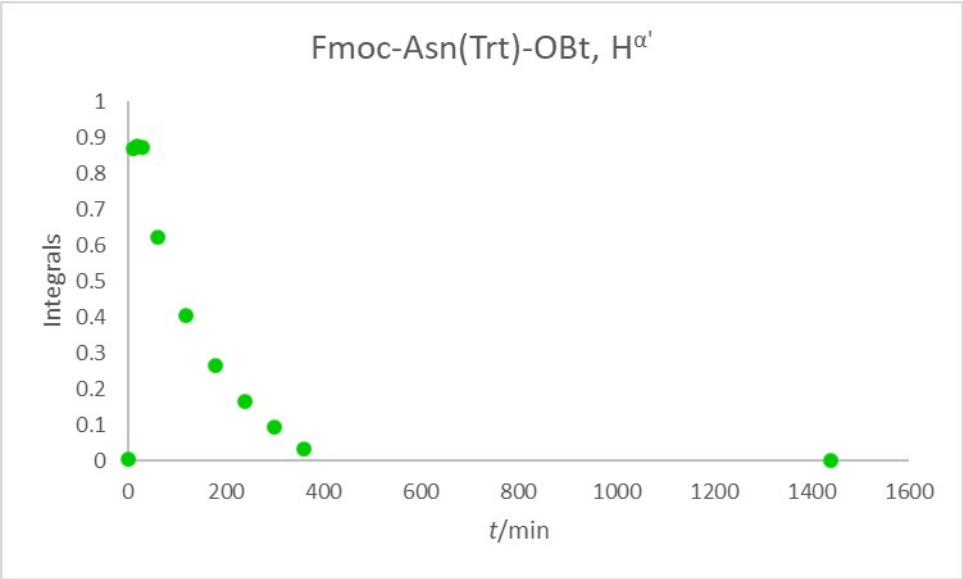


Figure 6. Integral-time diagram of Fmoc-Asn(Trt)-OBt active ester, Hα' signal at 5.21 ppm

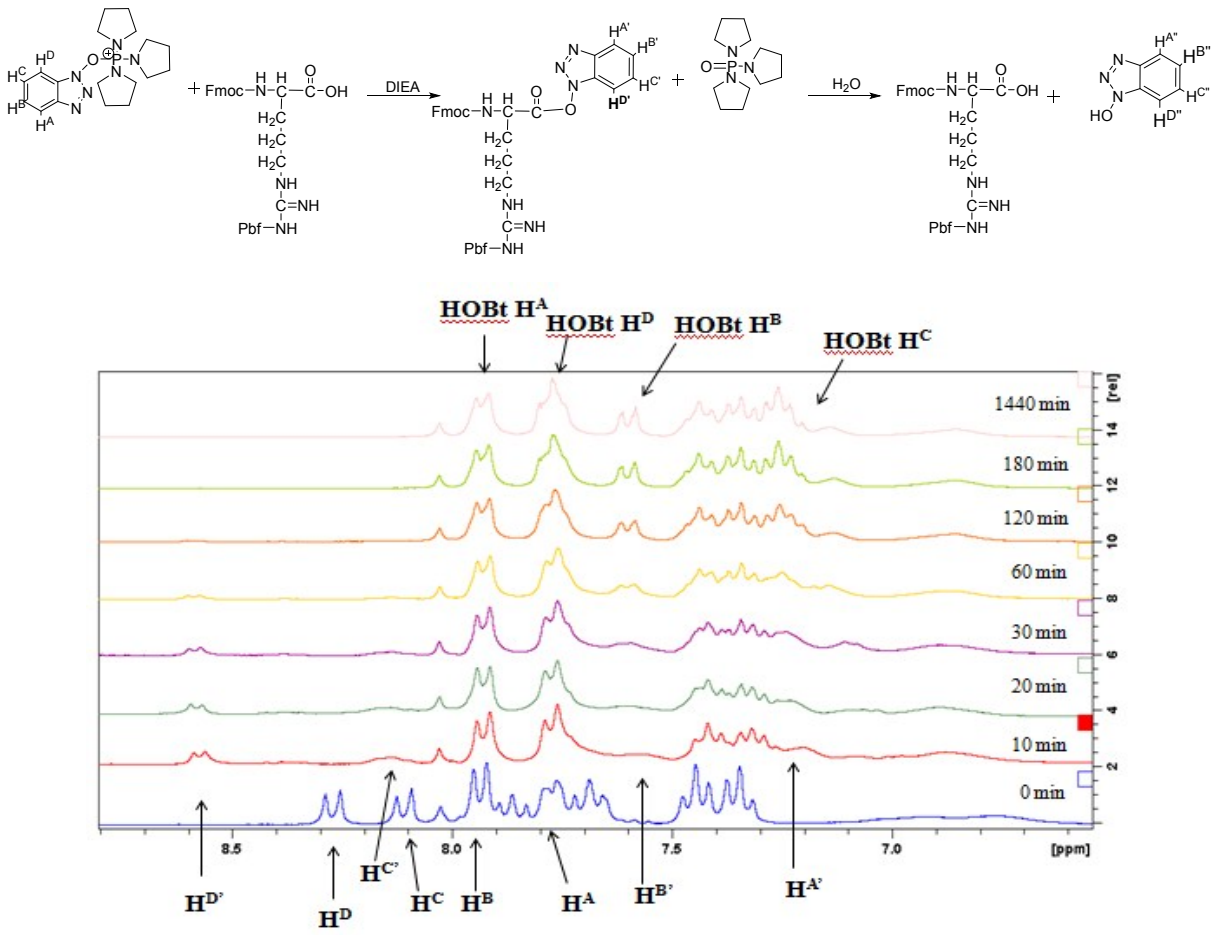


Figure 7. Reaction of Fmoc-Arg(Pbf)-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

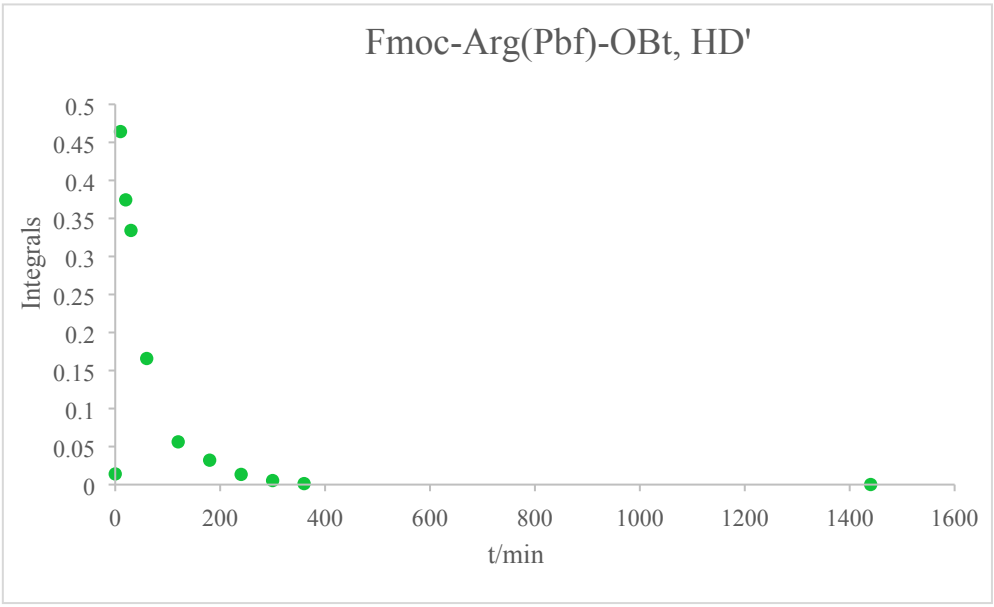


Figure 8. Integral-time diagram of Fmoc-Arg(Pbf)-OBt active ester, $H^{D'}$ signal at 8.62 ppm

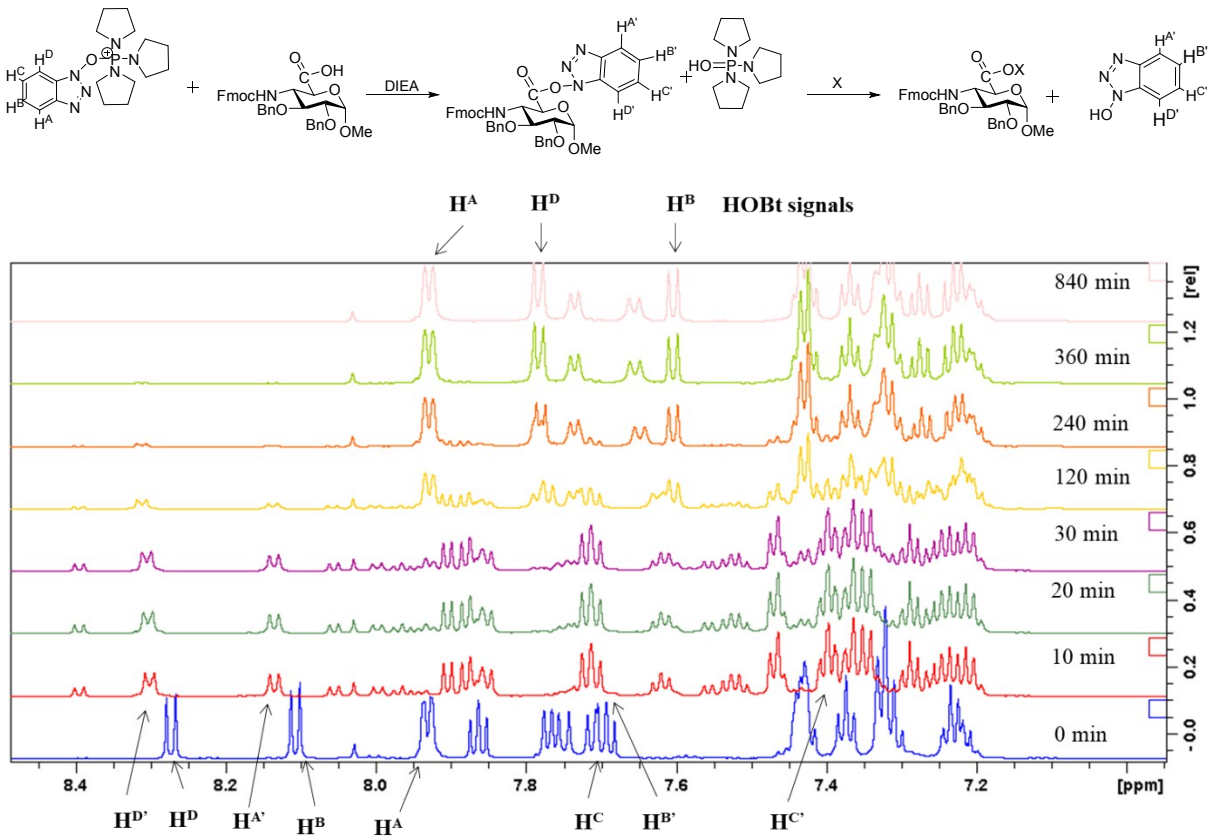


Figure 9. Reaction of Fmoc-GlcAPU(Me)-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 700 MHz

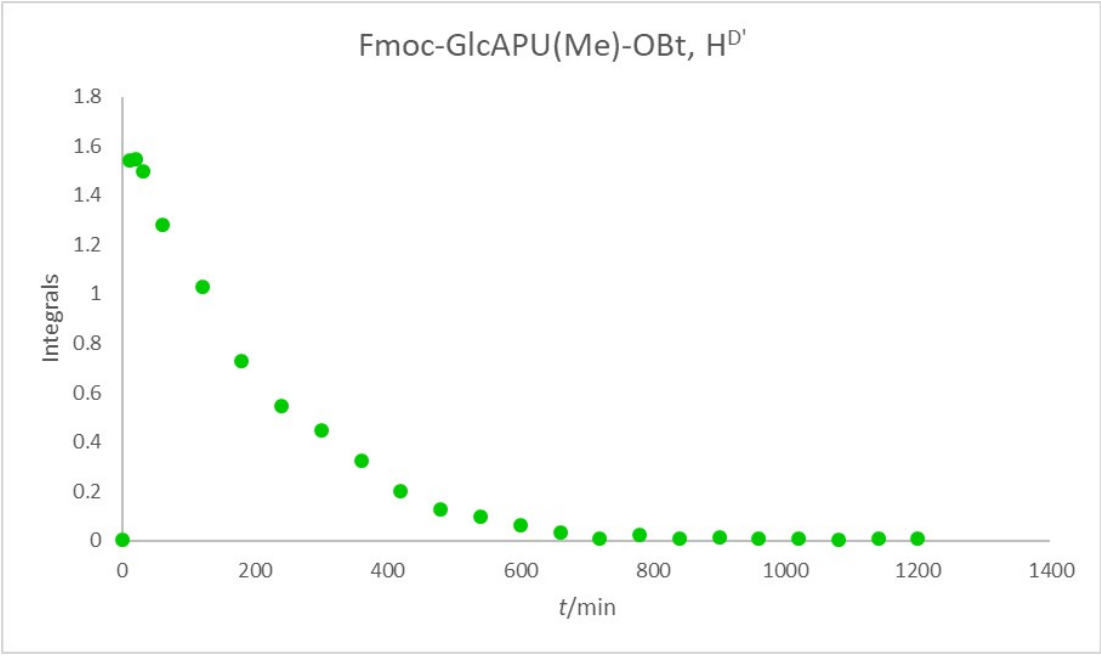
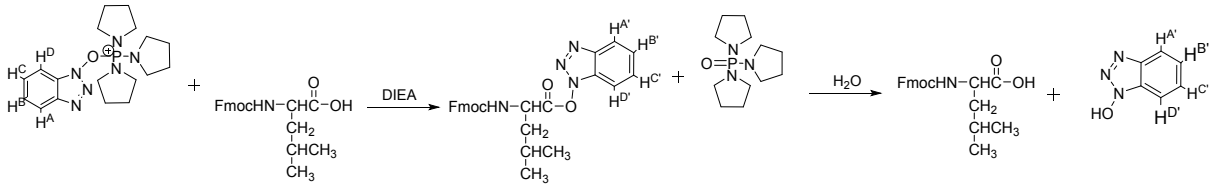


Figure 10. Integral-time diagram of Fmoc-GlcAPU(Me)-OBt active ester, $H^{D'}$ signal at 8.30 ppm

2.2. Slow hydrolyzing active esters



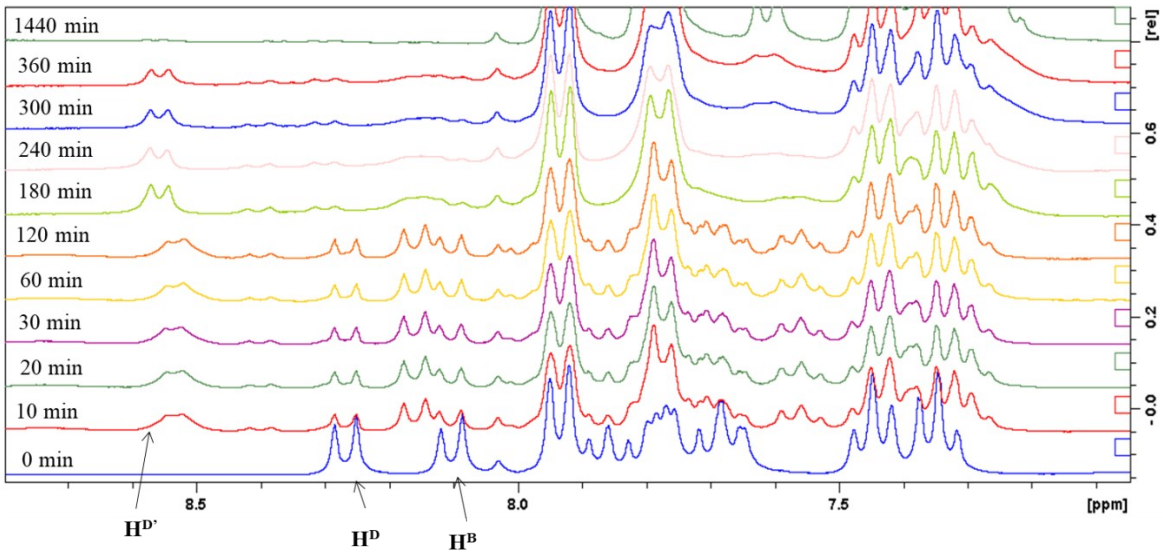


Figure 11. Reaction of Fmoc-Leu-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

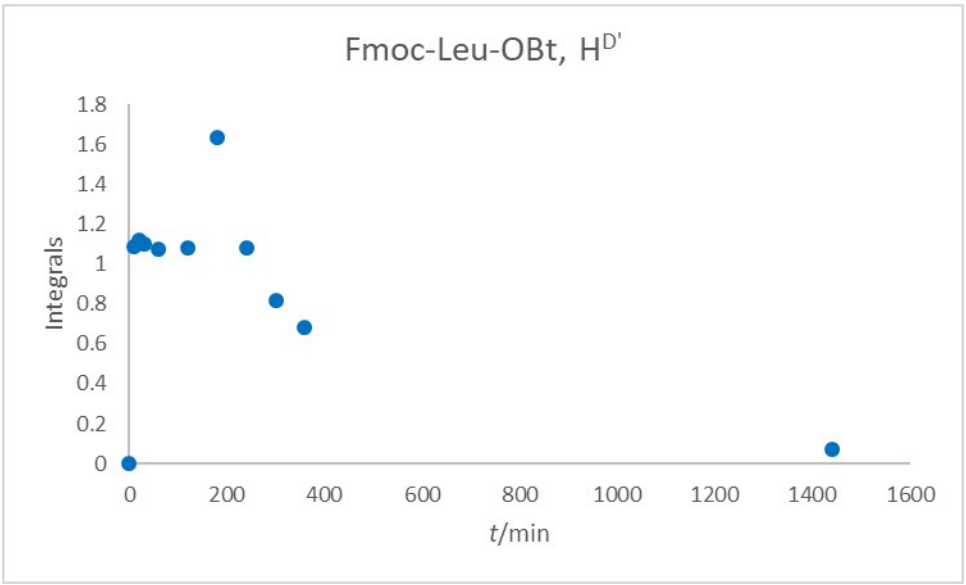
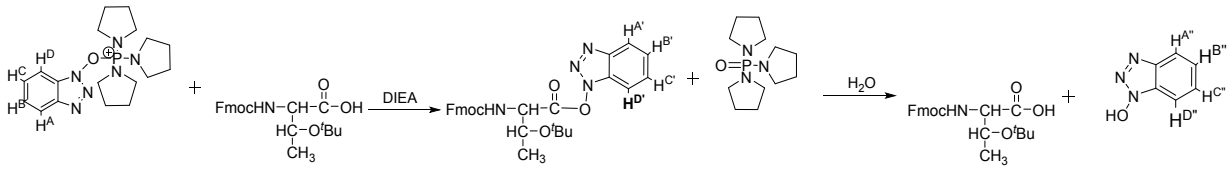


Figure 12. Integral-time diagram of Fmoc-Leu-OBt active ester, $H^{D'}$ signal at 8.53 ppm



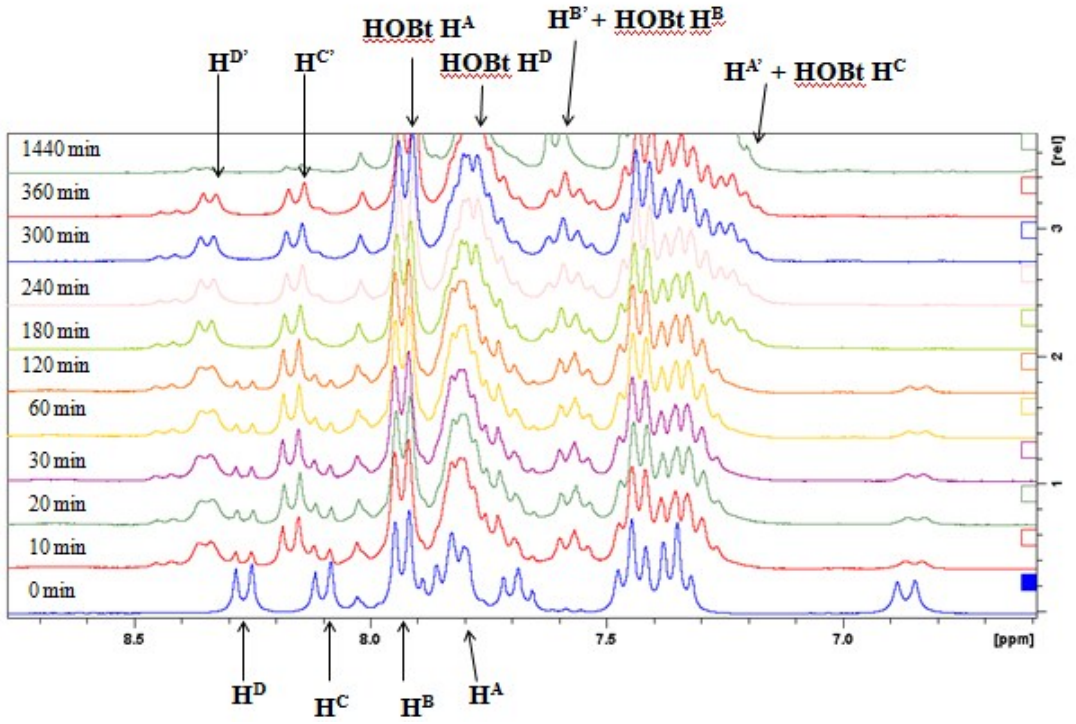


Figure 13. Reaction of Fmoc-Thr(ᵗBu)-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

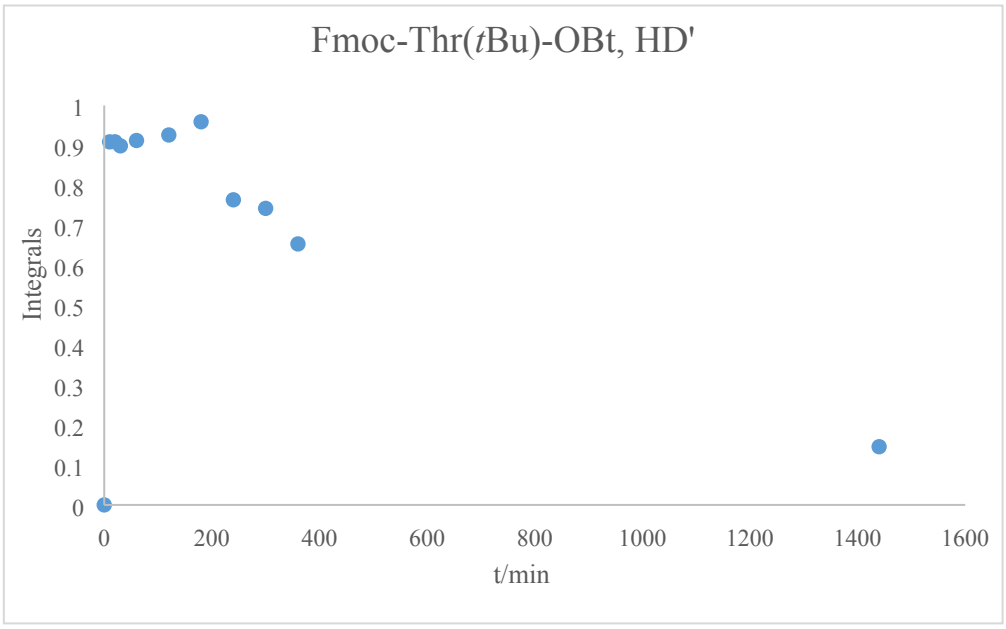
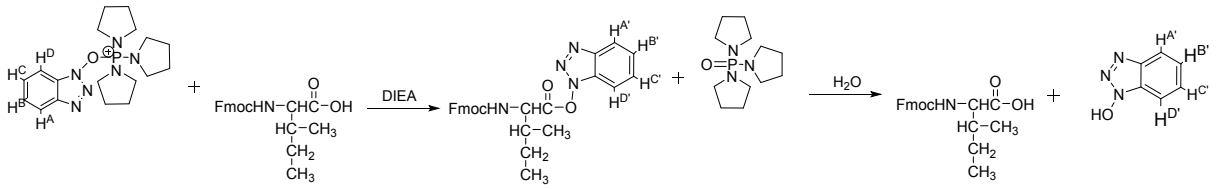


Figure 14. Integral-time diagram of Fmoc-Leu-OBt active ester, H^{D}' signal at 8.35 ppm



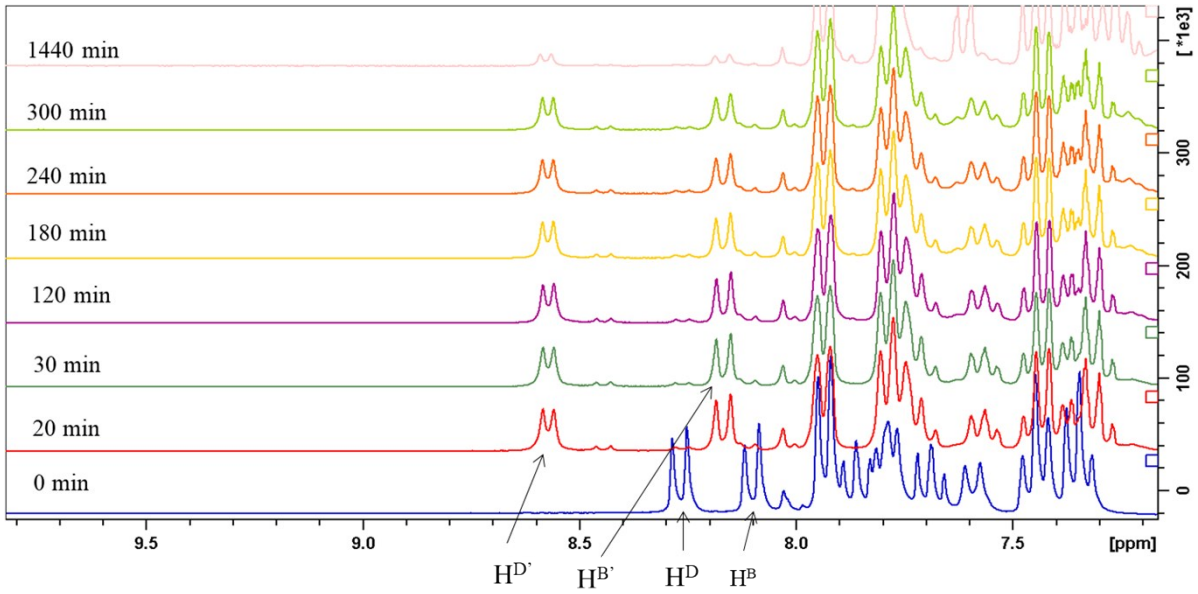


Figure 15. Reaction of Fmoc-Ile-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

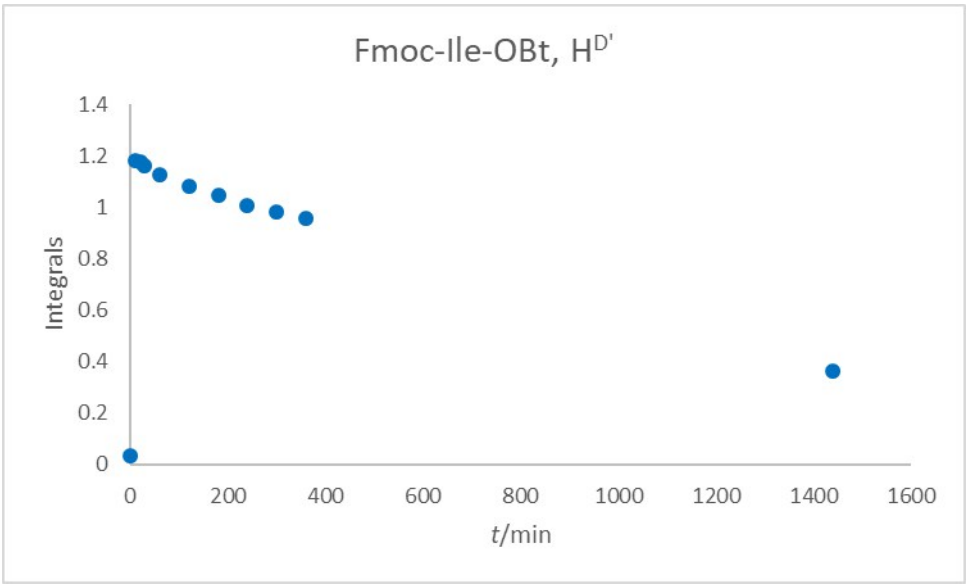
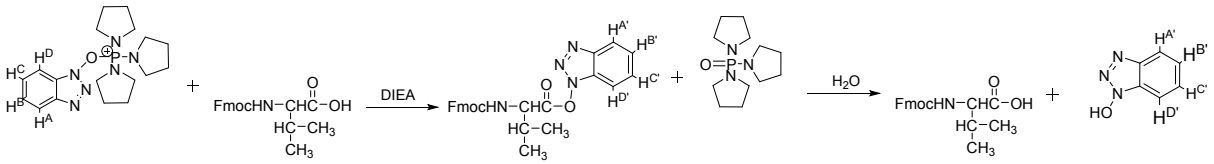


Figure 16. Integral-time diagram of Fmoc-Ile-OBt active ester, $H^{D'}$ signal at 8.57 ppm



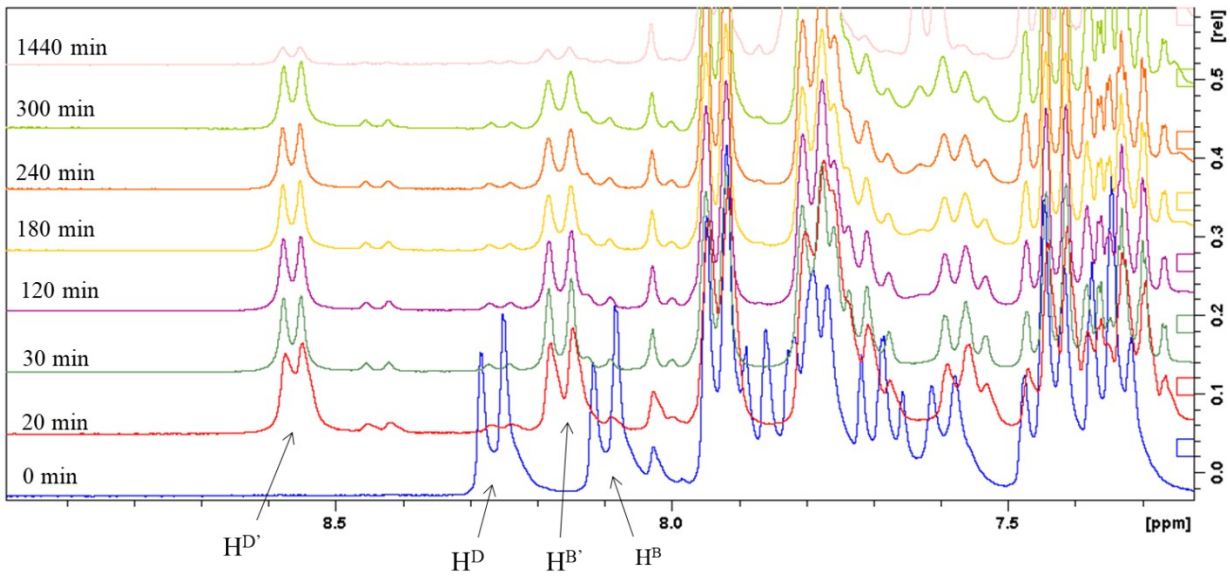


Figure 17. Reaction of Fmoc-Val-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

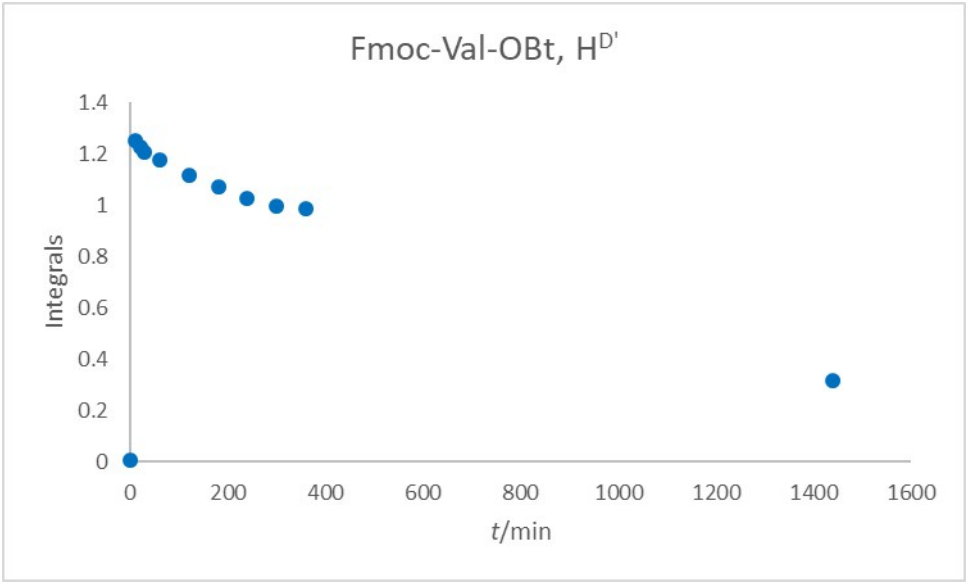


Figure 18. Integral-time diagram of Fmoc-Val-OBt active ester, $H^{D'}$ signal at 8.56 ppm

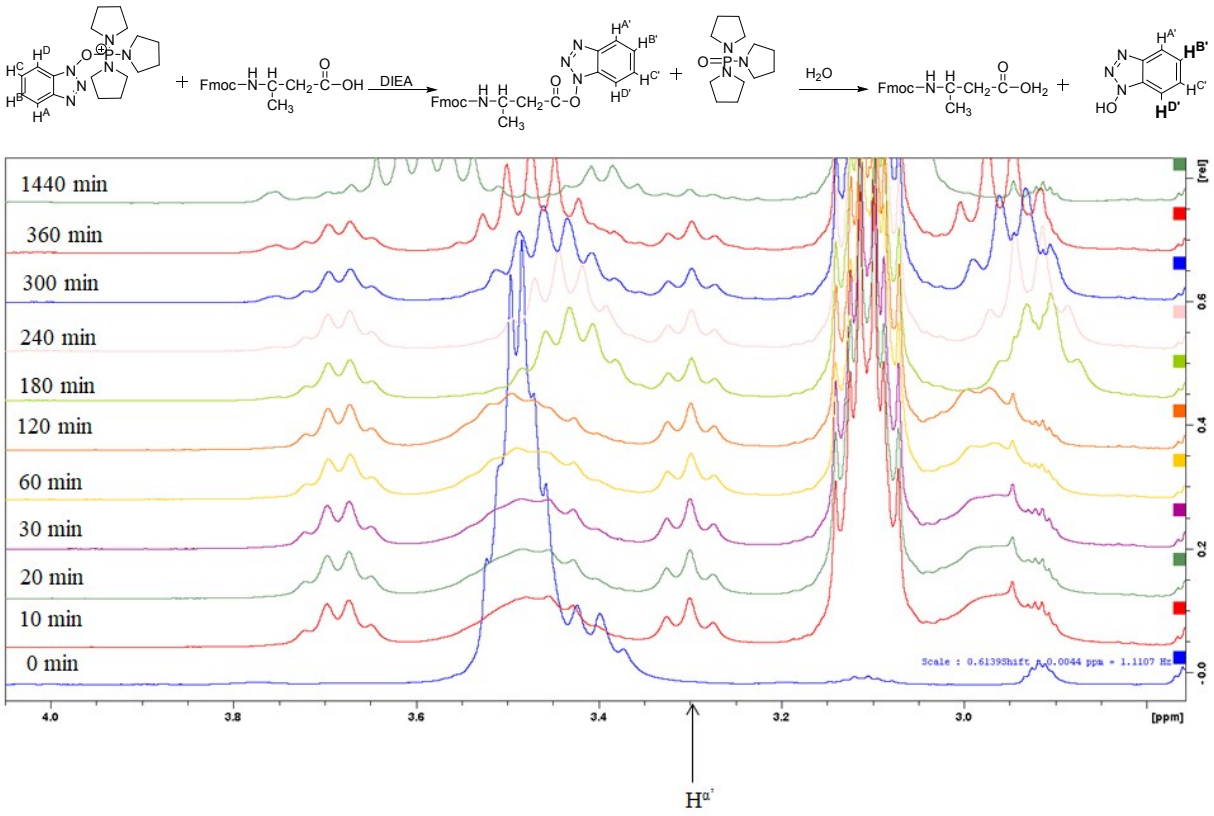


Figure 19. Reaction of Fmoc-β-Ala-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aliphatic region, 250 MHz

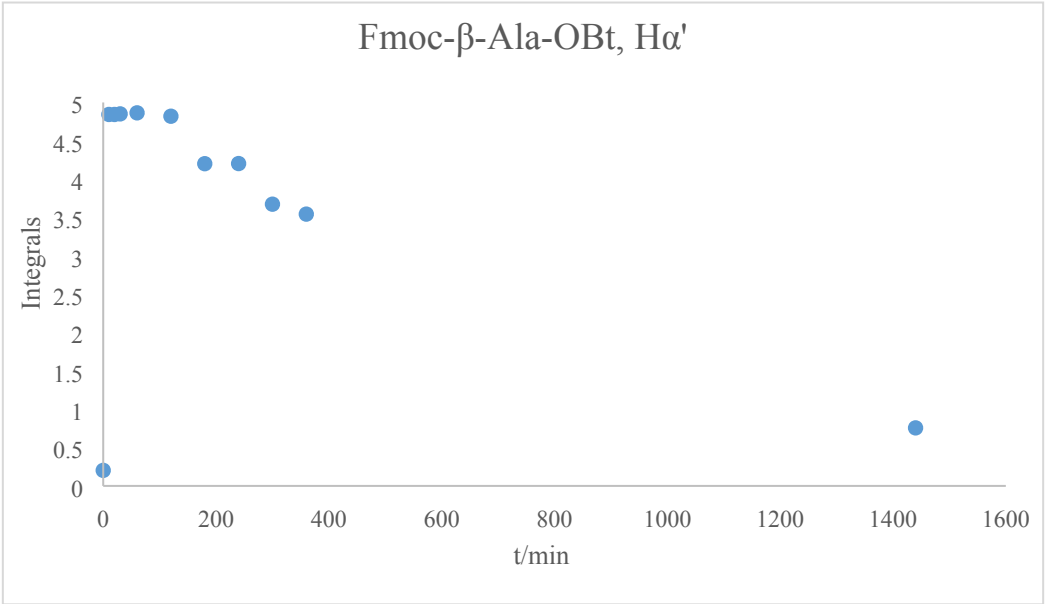


Figure 20. Integral-time diagram of Fmoc-β-Ala-OBt active ester, Hα' signal at 3.3 ppm

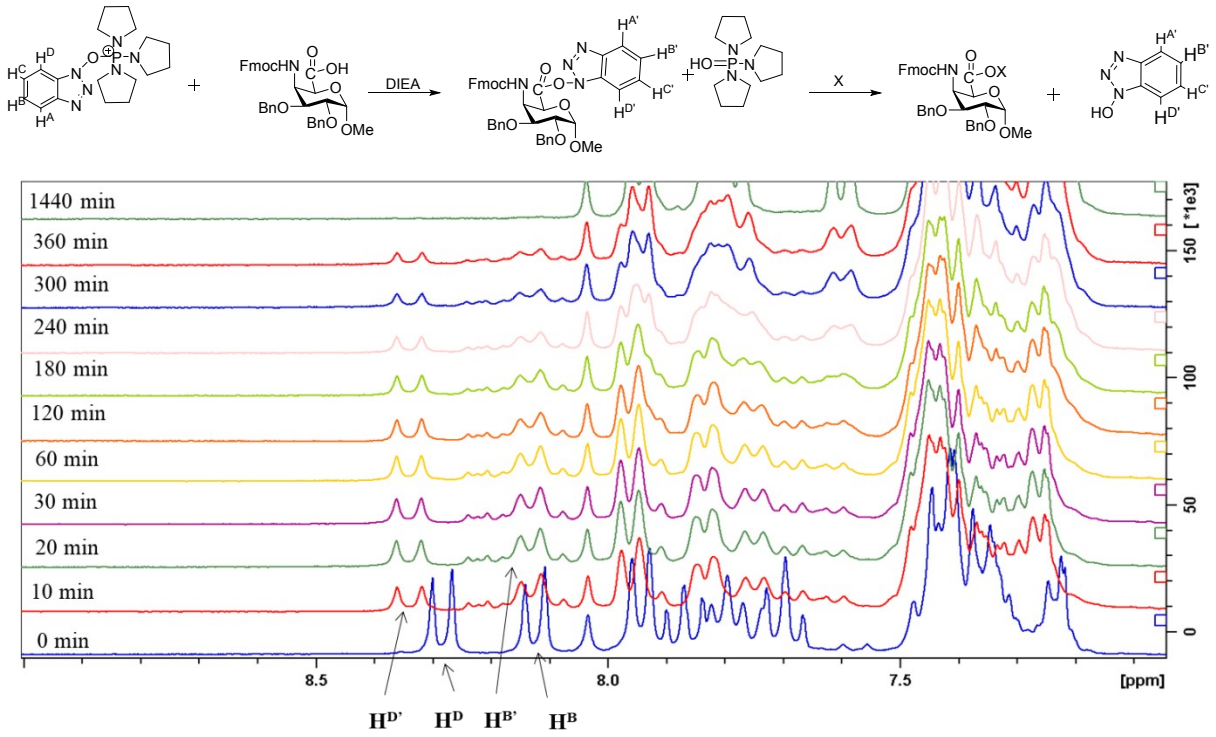


Figure 21. Reaction of Fmoc-GalAPU(Me)-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

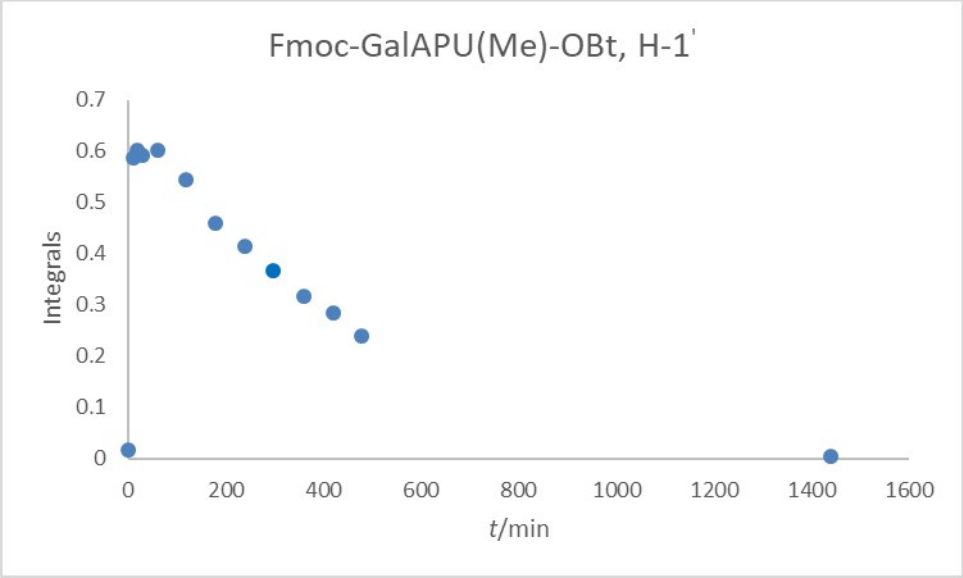
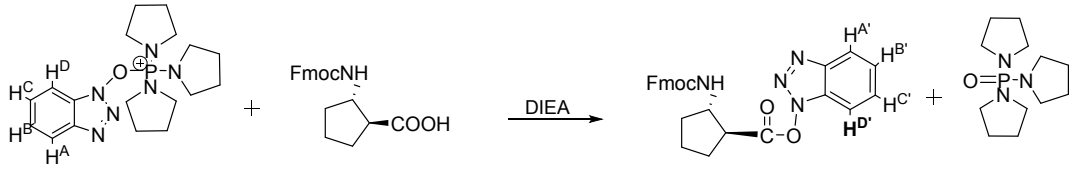


Figure 22. Integral-time diagram of Fmoc-GalAPU(Me)-OBt active ester, H-1' signal at 5.53 ppm

2.3. None hydrolyzing active esters



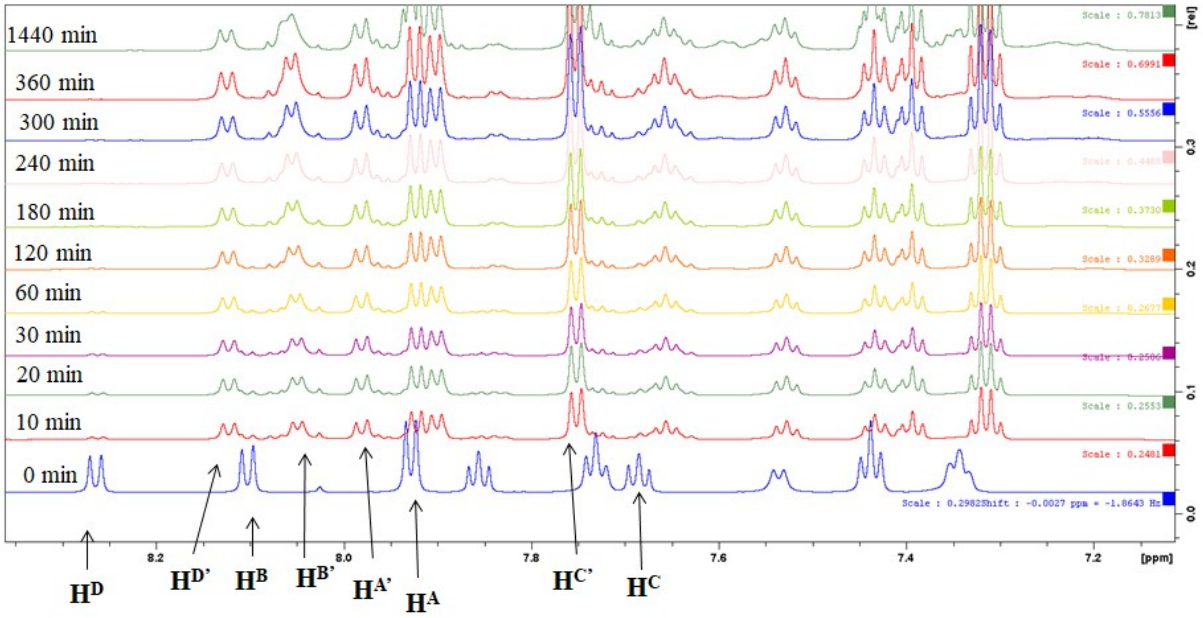


Figure 23. Reaction of Fmoc-ACPC-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 700 MHz

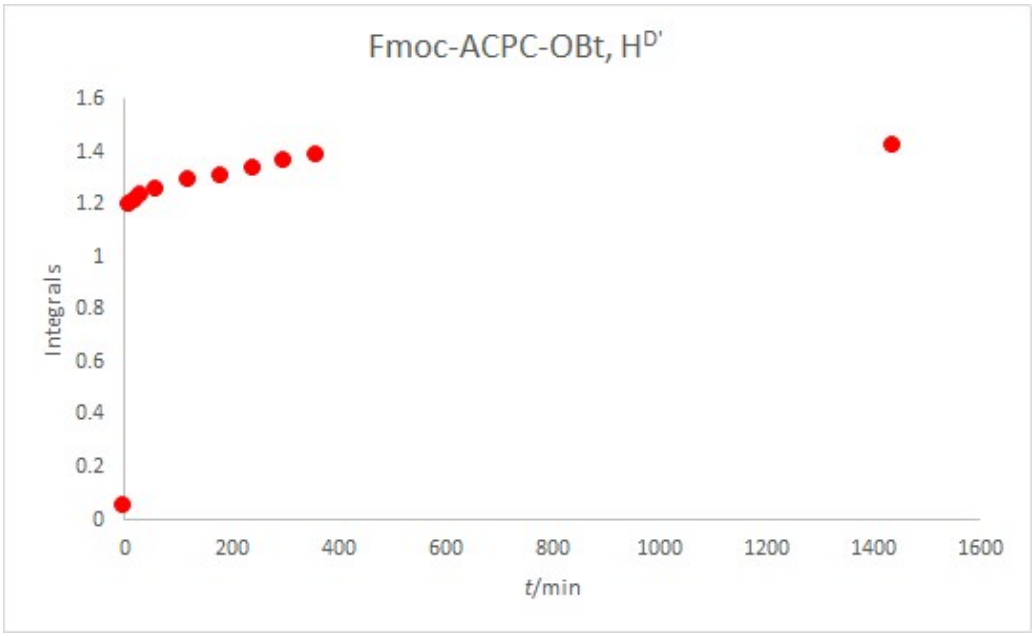


Figure 24. Integral-time diagram of Fmoc-ACPC-OBt active ester, H$^{D'}$ signal at 8.13 ppm

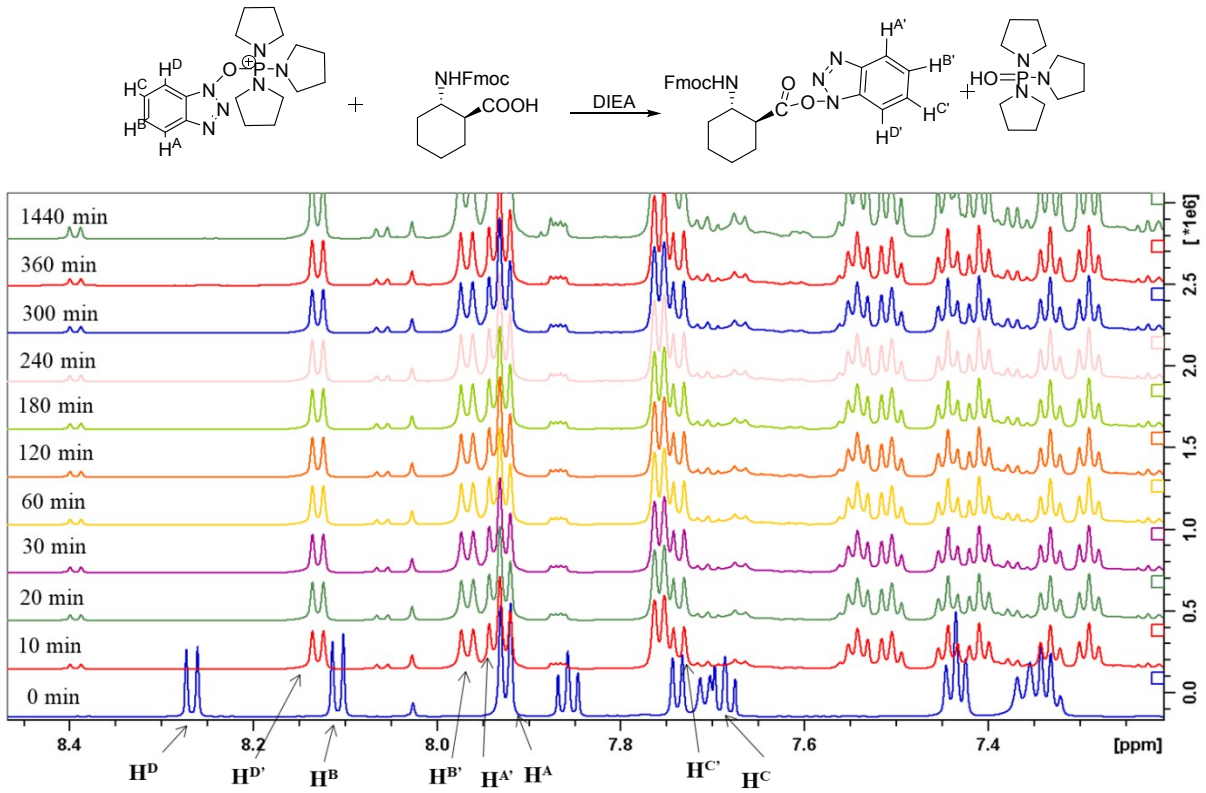


Figure 25. Reaction of Fmoc-ACHC-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 700 MHz

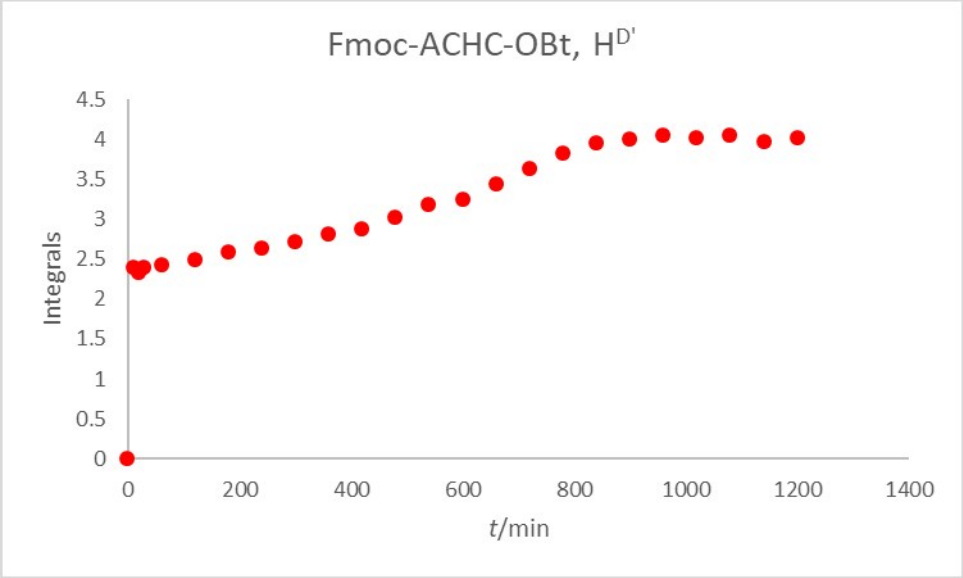


Figure 26. Integral-time diagram of Fmoc-ACPC-OBt active ester, H-1' signal at 8.13 ppm

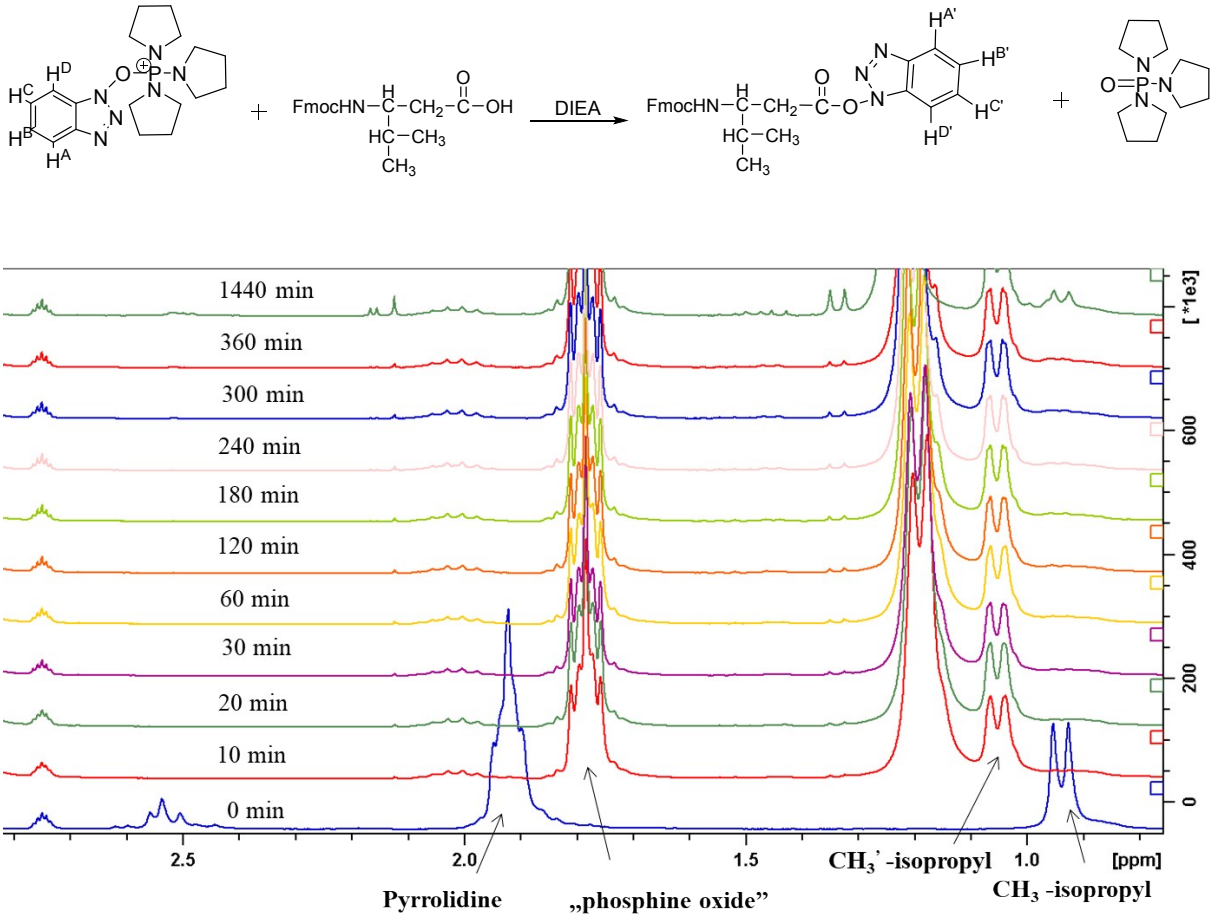


Figure 27. Reaction of Fmoc-β^3-Val-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, side-chain region, 250 MHz

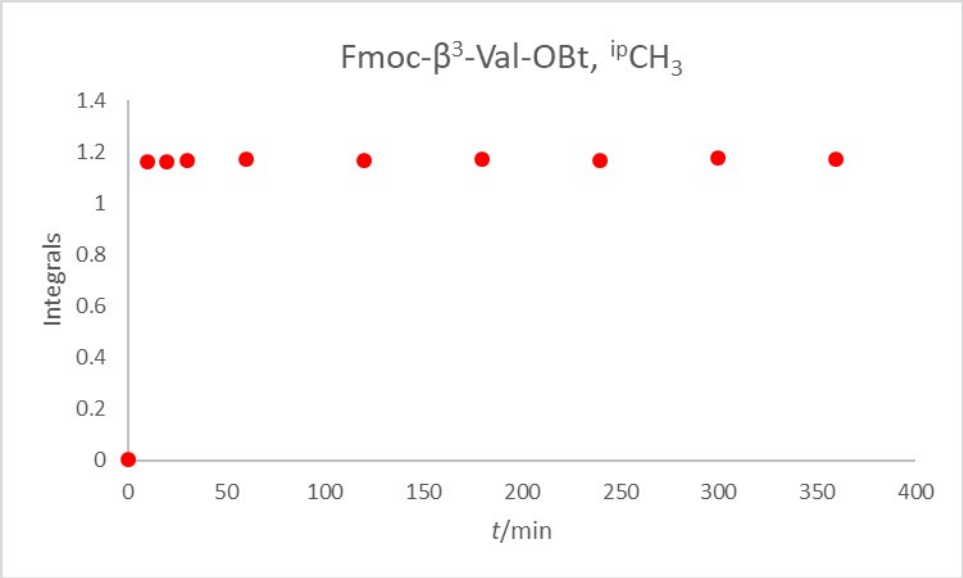
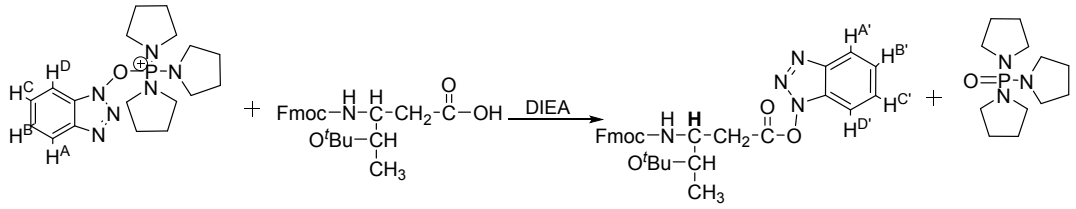


Figure 28. Integral-time diagram of Fmoc-β^3-Val-OBt active ester, $^{ip}CH_3$ signal at 1.04 ppm



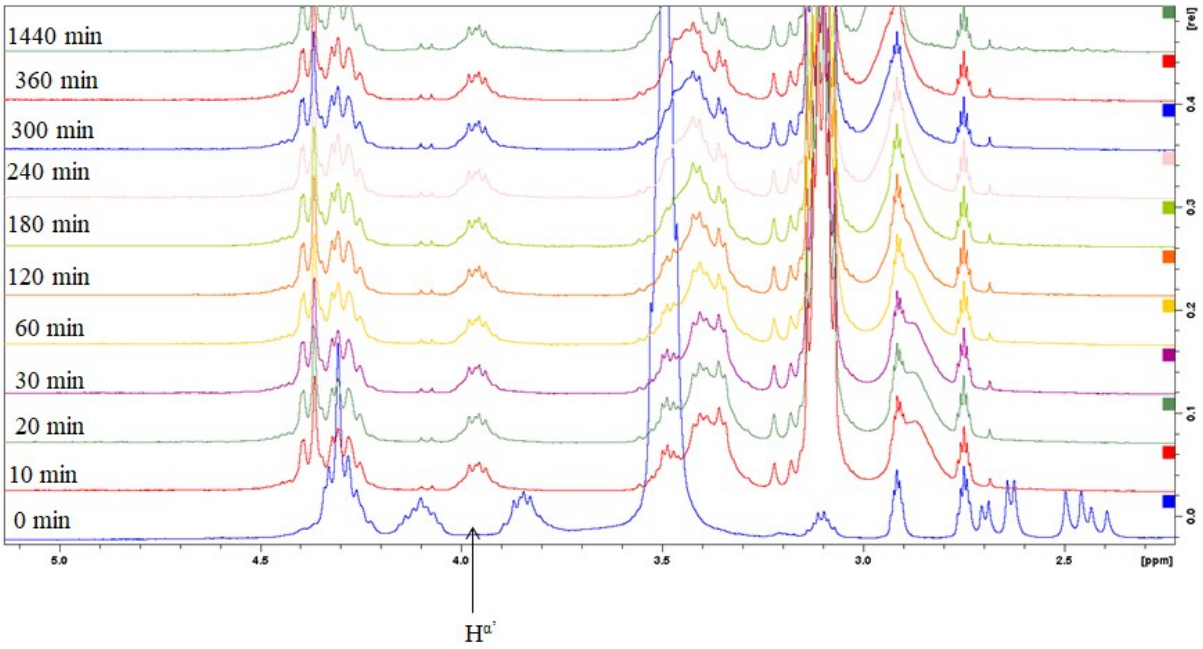


Figure 29. Reaction of Fmoc-β³-Thr(ᵗBu)-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aliphatic region, 250 MHz

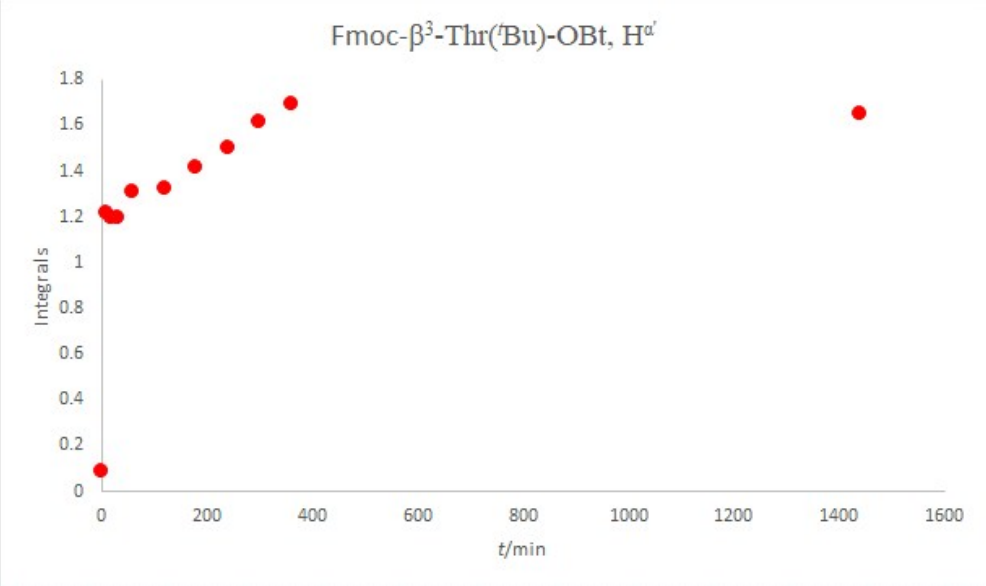


Figure 30. Integral-time diagram of Fmoc-β³-Thr(ᵗBu)-OBt active ester, Hα' signal at 3.98 ppm

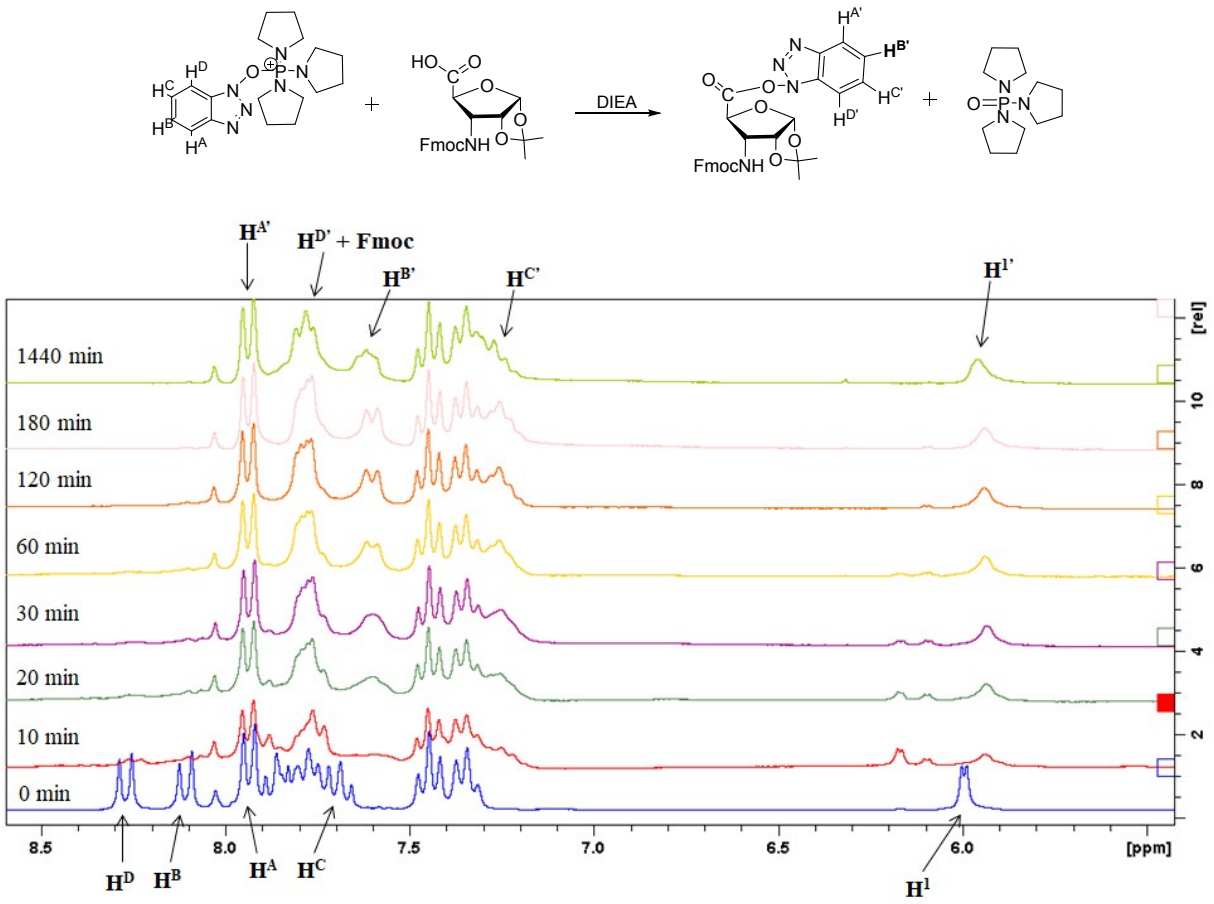


Figure 31. Reaction of Fmoc-RibAFU(ip)-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

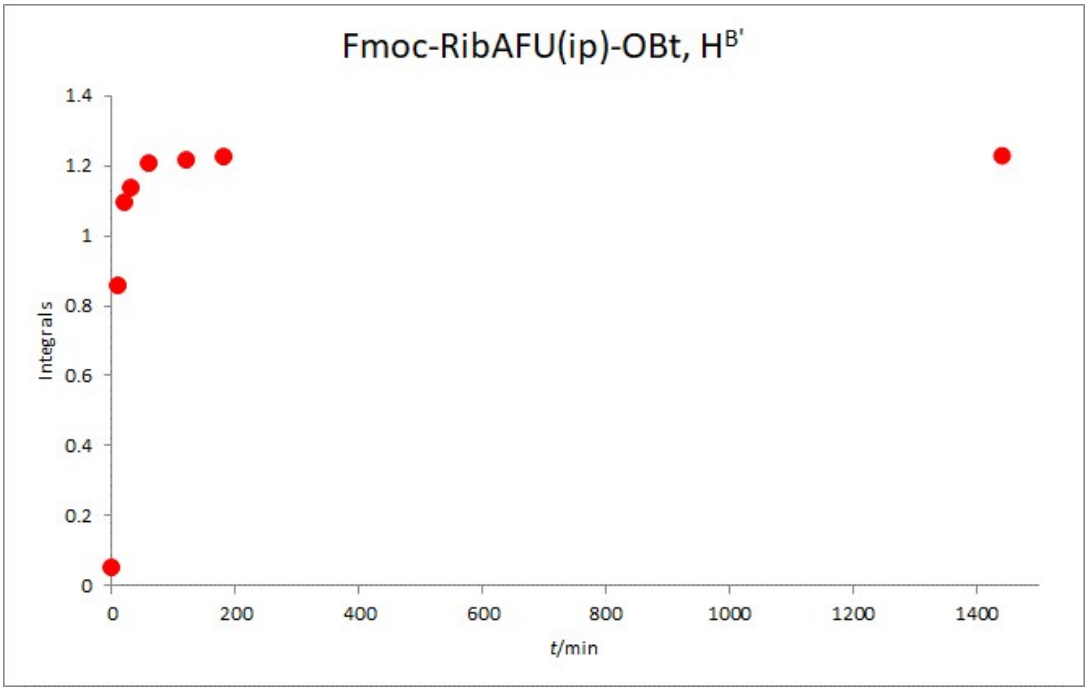


Figure 32. Integral-time diagram of Fmoc-RibAFU(ip)-OBt active ester, $H^{B'}$ signal at 7.61 ppm

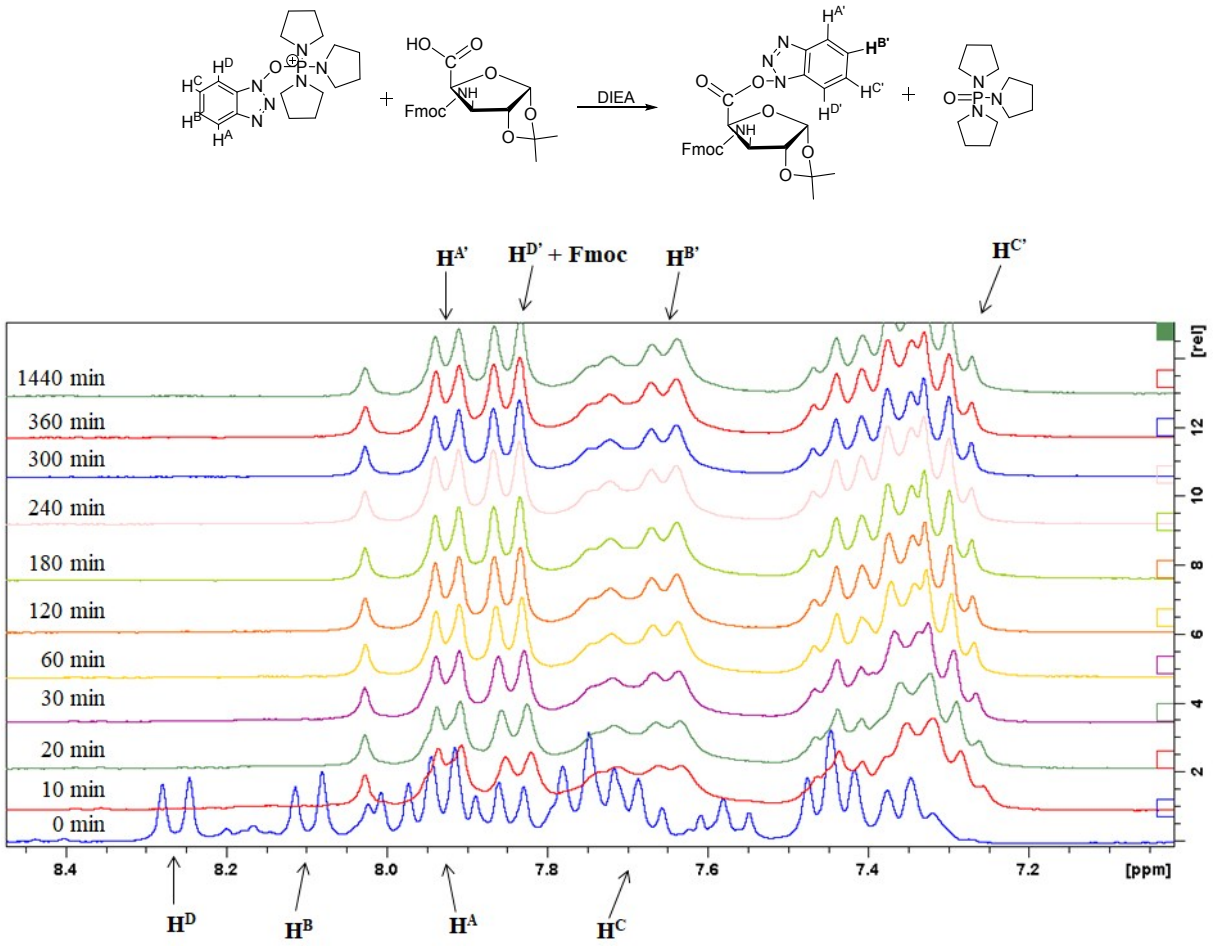


Figure 33. Reaction of Fmoc-XylAFU(ip)-OH and PyBOP/DIEA in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

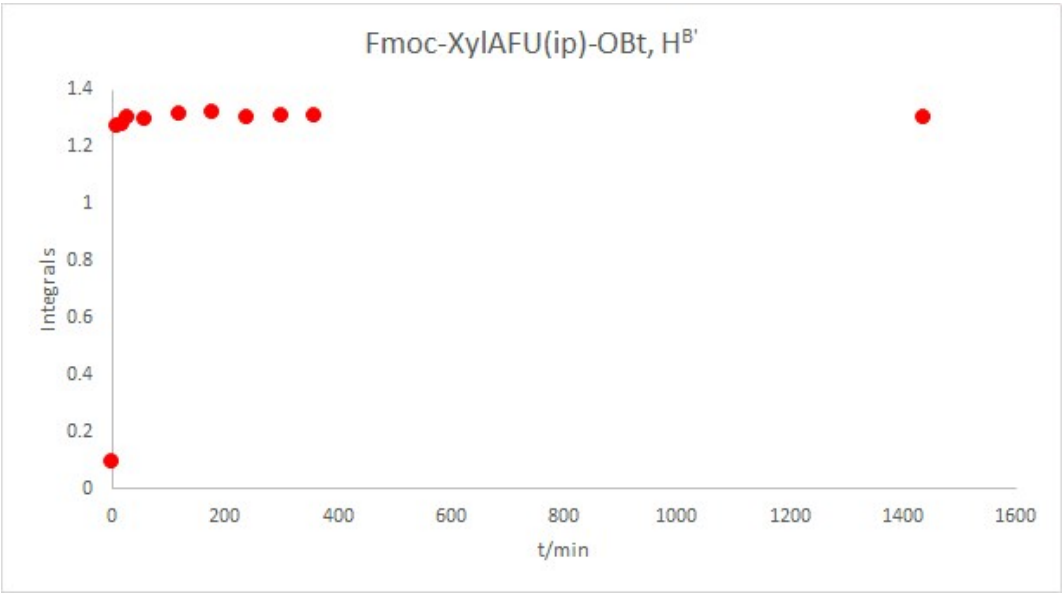


Figure 34. Integral-time diagram of Fmoc-XylAFU(ip)-OBt active ester, $H^{B'}$ signal at 7.65 ppm

3. ^{1}H-NMR Spectra and diagrams of the active esters formation and hydrolysis with HOBt/DIC

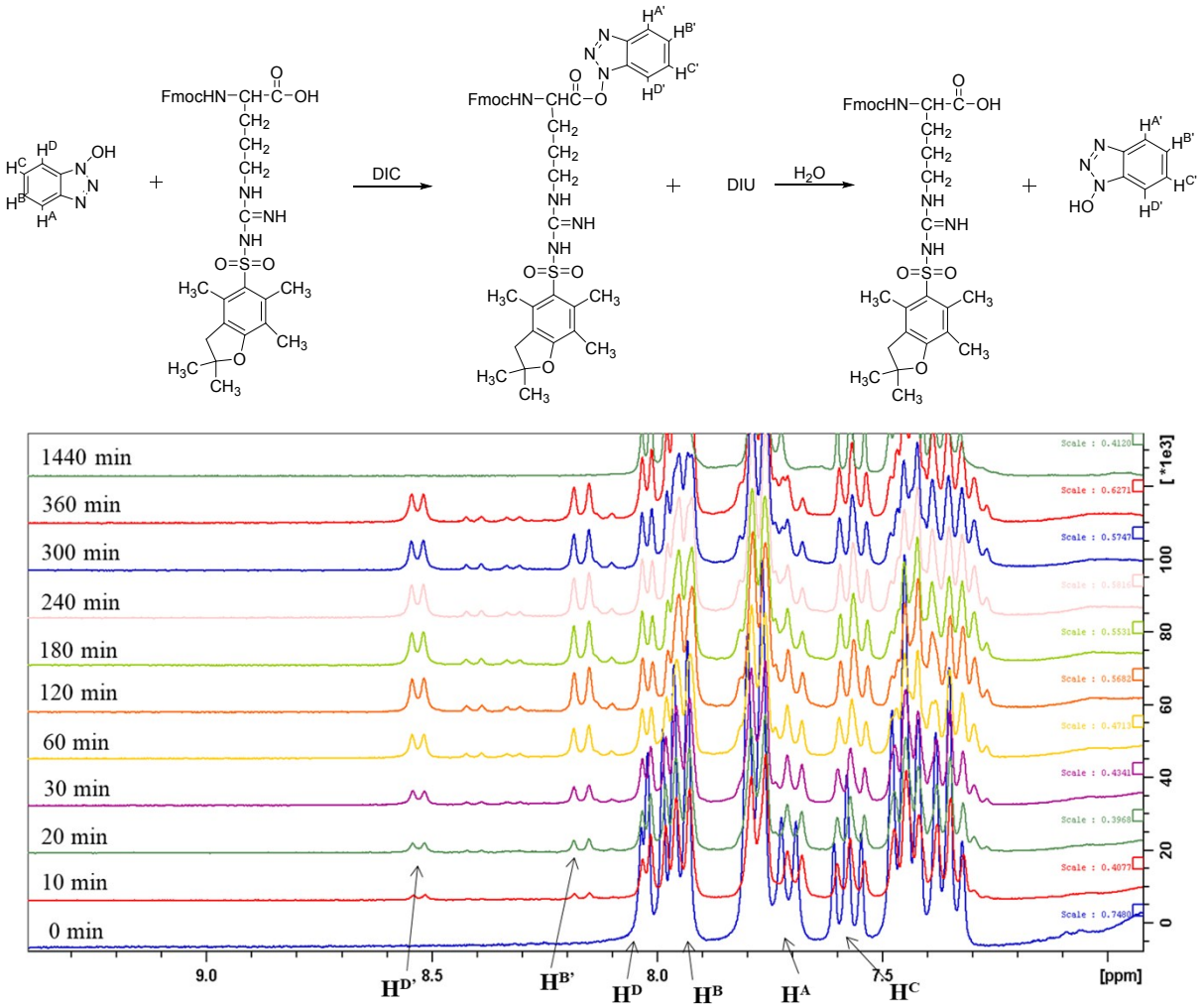


Figure 35. Reaction of Fmoc-Arg(Pbf)-OH and DIC/HOBt in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

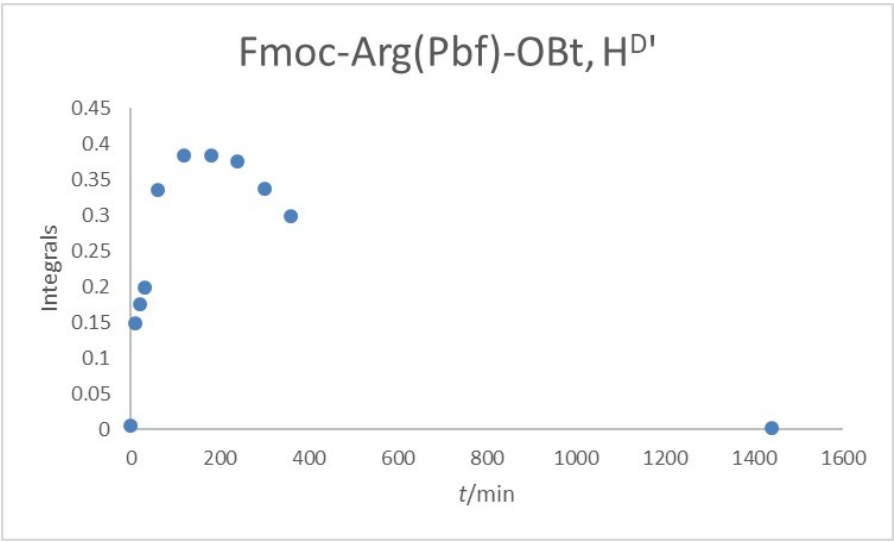
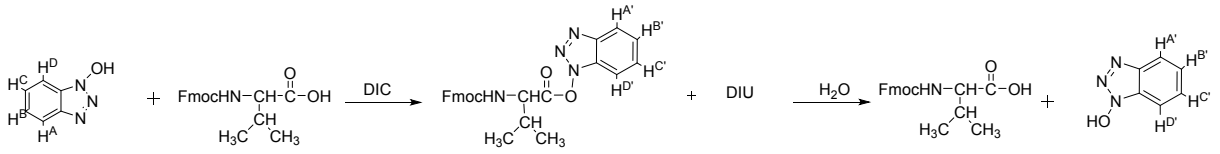


Figure 36. Integral-time diagram of Fmoc-Arg(Pbf)-OBt active ester, $H^{D'}$ signal at 8.52 ppm



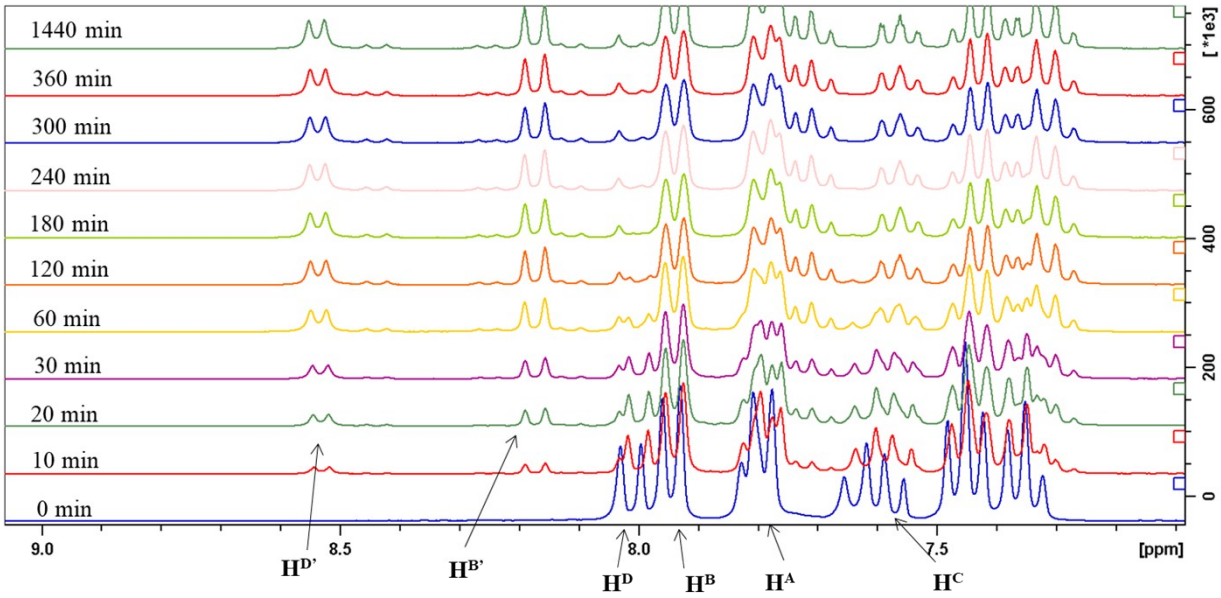


Figure 37. Reaction of Fmoc-Val-OH and DIC/HOBt in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

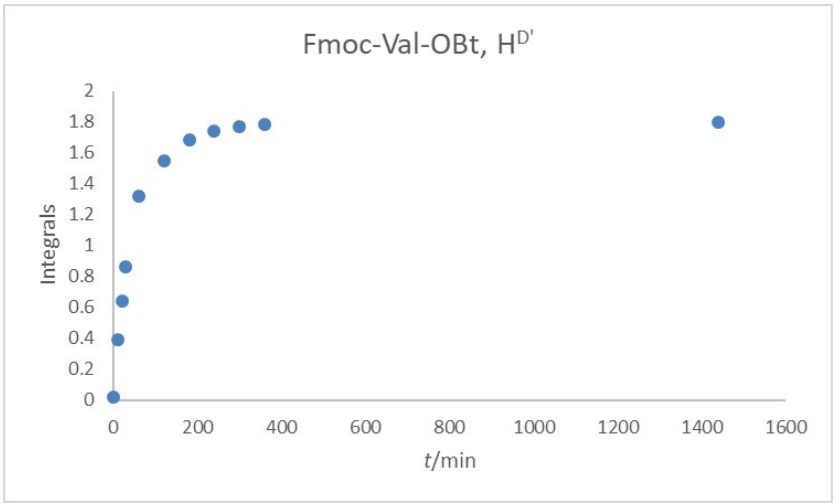


Figure 38. Integral-time diagram of Fmoc-Val-OBt active ester, $H^{D'}$ signal at 8.54 ppm

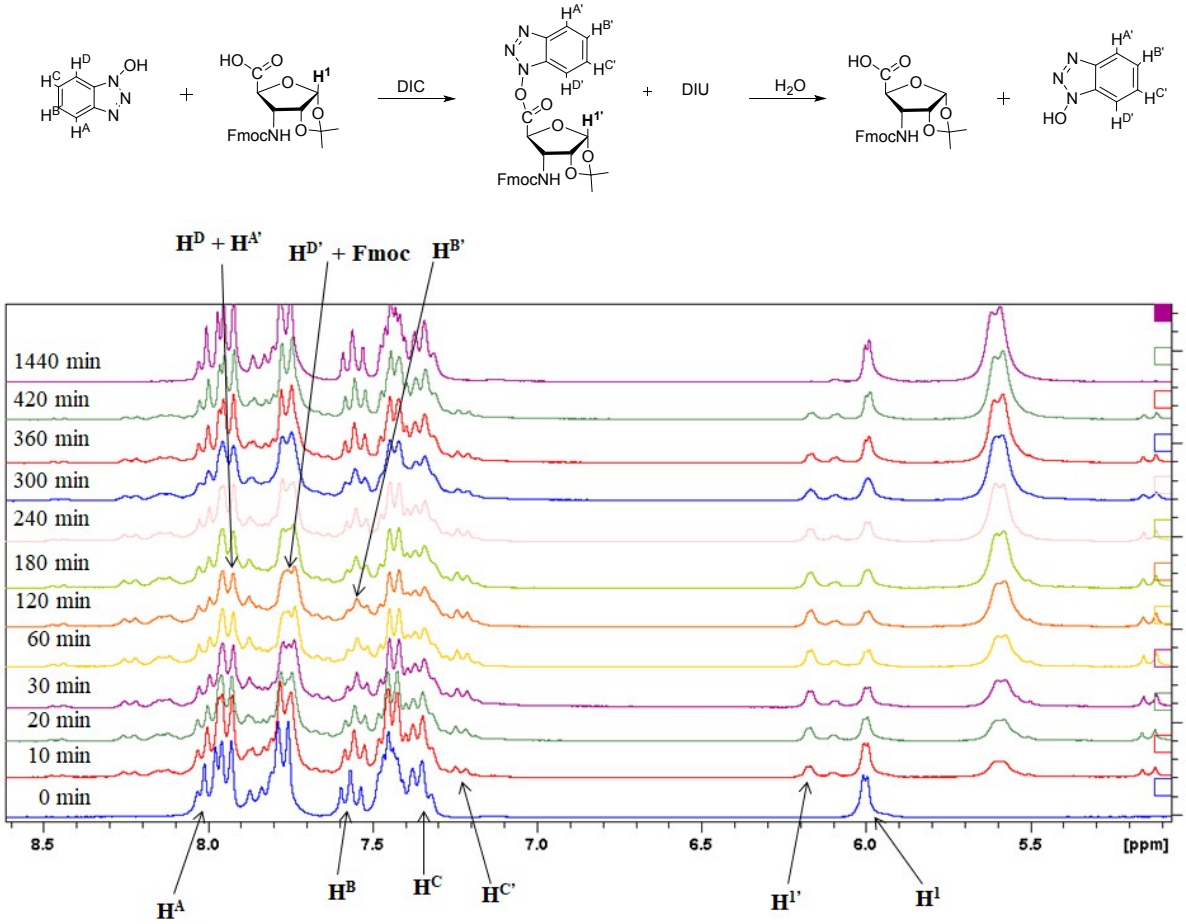


Figure 39. Reaction of Fmoc-RibAFU(ip)-OH and DIC/HOBt in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

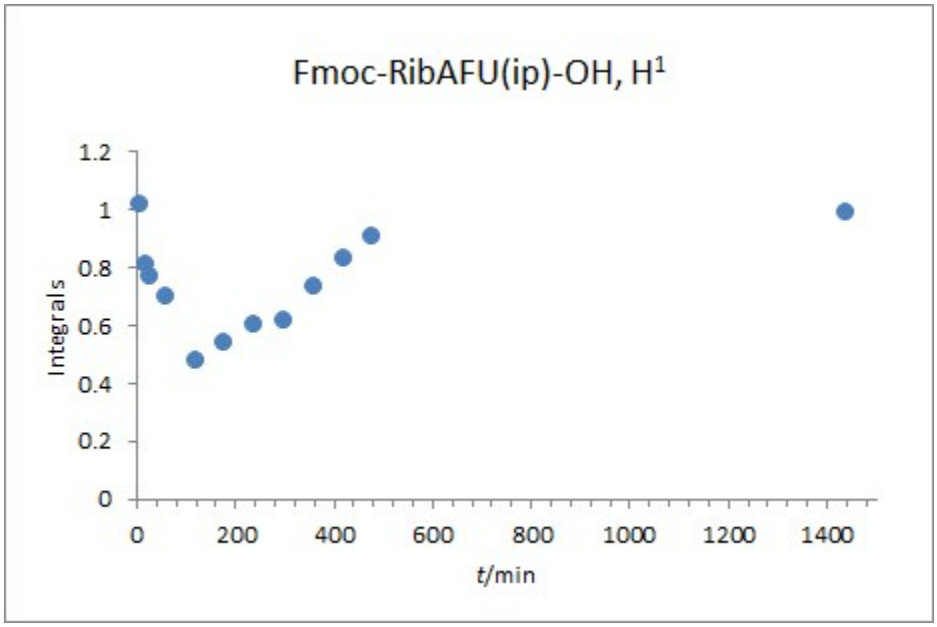


Figure 40. Integral-time diagram of Fmoc-RibAFU(ip)-OBt active ester, H^{1}' signal at 6.19 ppm

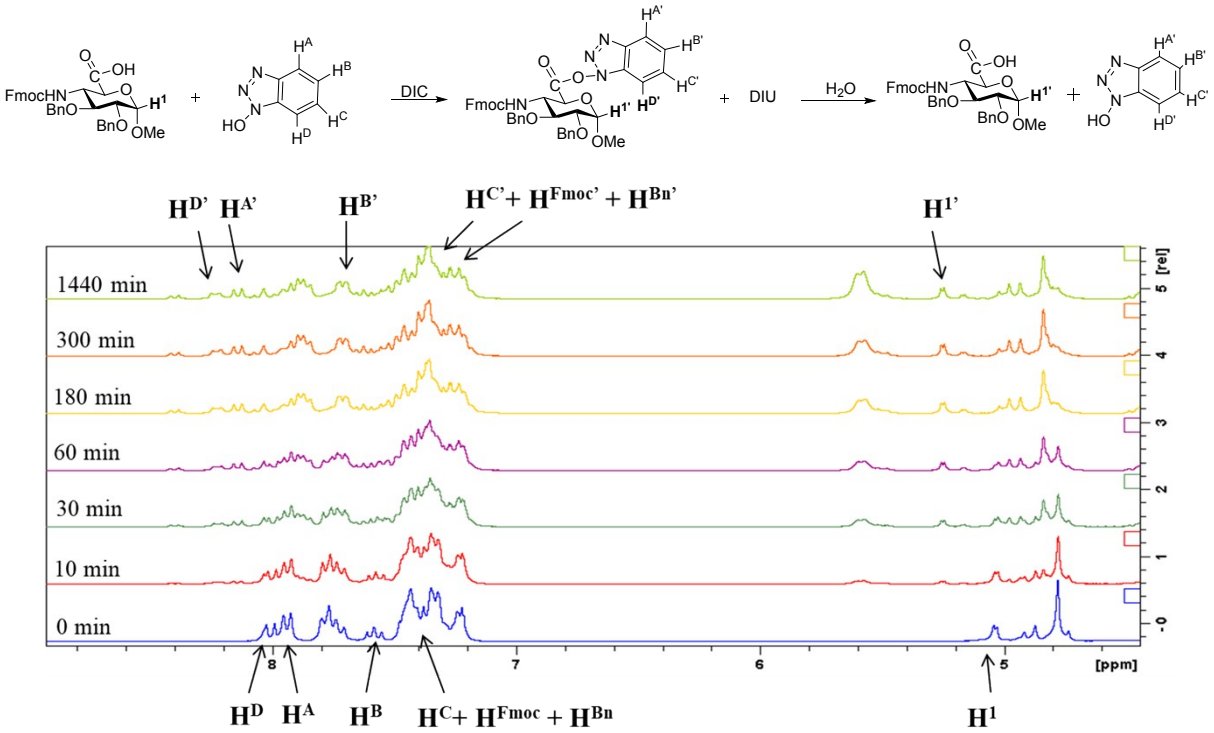


Figure 41. Reaction of Fmoc-GlcAPU(Me)-OH and DIC/HOBt in DMF-d_7 as a function of time; ^{1}H NMR spectra, aromatic region, 250 MHz

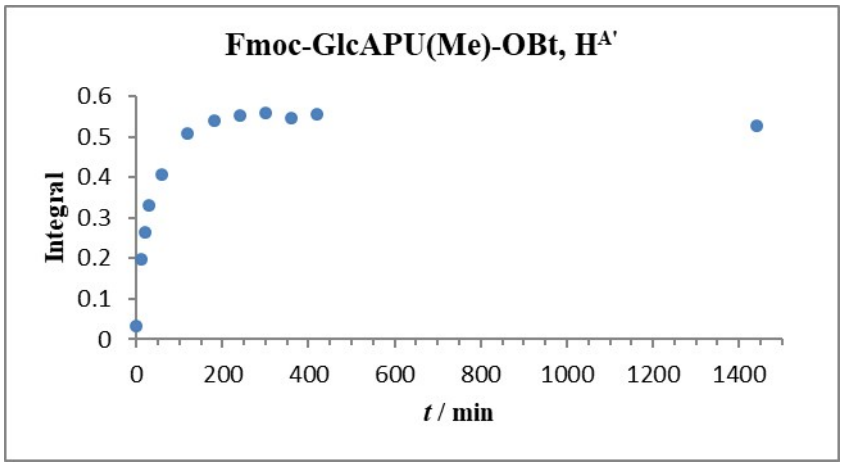


Figure 42. Integral-time diagram of Fmoc-GlcAPU(Me)-OBt active ester, H^{1}' signal at 8.08 ppm

4. Kinetic analysis

Table 2. Parameter Estimation Summary: calculated parameters and their confidence intervals estimated using the COPASI 4.16 (Build 104)

parameter	ndf[a]	t (0.975)	$k_{(hydrolysis)}$					$[AC]_{0,calc}$					$[H2O]_0$				
amino acis			**Value**	Std. dev.	**int. hw[b]**	low	high	**Value**	Std. dev.	**int. hw**	low	high	**Value**	Std. dev.	**int. hw**	low	high
Fmoc-Ile-OH	3	4.177	**0.0031**	0.0715	**0.2987**	-0.2956	0.3018	**0.068**	0.0012	**0.0050**	0.063	0.073	**0.226**	5.142	**21.475**	-21.249	21.702
Fmoc-Val-OH	5	3.163	**0.0460**	0.0046	**0.0145**	0.0315	0.0605	**0.095**	0.0003	**0.0008**	0.094	0.096	**0.029**	0.001	**0.004**	0.024	0.033
Fmoc-Arg(Pbf)-OH	6	2.969	**0.1098**	0.2170	**0.6443**	-0.5345	0.7541	**0.021**	0.0007	**0.0022**	0.019	0.023	**0.184**	0.340	**1.011**	-0.827	1.195
Fmoc-Ala-OH	7	2.841	**0.0246**	0.0014	**0.0040**	0.0206	0.0286	**0.110**	0.0023	**0.0066**	0.103	0.116	cannot be estimated; fixed at 0.25 mM				
Fmoc-Asn(Trt)-OH	7	2.841	**0.0447**	3.87E-05	**0.0001**	0.0446	0.0448	**0.071**	0.0042	**0.0119**	0.060	0.083	**0.229**	0.156	**0.444**	-0.215	0.672
Fmoc-Leu-OH	4	3.495	**0.0504**	0.0068	**0.0237**	0.0267	0.0742	**0.069**	0.00081	**0.0028**	0.066	0.071	**0.093**	0.008	**0.029**	0.064	0.123
Fmoc-GlcAPU(Me)-OH	8	2.752	**0.0224**	0.0007	**0.0020**	0.0205	0.0244	**0.059**	0.00094	**0.0026**	0.056	0.061	cannot be estimated; fixed at 0.25 mM				
Fmoc-Thr(^{t}Bu)-OH	3	4.177	**0.0248**	0.0028	**0.0117**	0.0131	0.0365	**0.059**	0.00038	**0.0016**	0.057	0.061	**0.095**	0.00757	**0.032**	0.064	0.127
Fmoc-βAla-OH	2	6.205	**0.0073**	0.00066	**0.0041**	0.0032	0.0114	**0.136**	0.0026	**0.0162**	0.120	0.152	cannot be estimated; fixed at 0.25 mM				
Fmoc-GalAPU(Me)-OH	6	2.969	**0.0092**	0.00027	**0.0008**	0.0084	0.0100	**0.060**	0.0011	**0.0034**	0.056	0.063	cannot be estimated; fixed at 0.25 mM				
Fmoc-Gly-OH	8	2.752	**0.0449**	0.0097	**0.0266**	0.0183	0.0715	**0.102**	0.0013	**0.0036**	0.098	0.106	**0.1423**	0.020151	**0.055**	0.087	0.198

a) is the number of degrees of freedom of the associated Student distribution calculated as the number of data – the number of parameters.

b) always stands for half width of the 95% confidence interval

Some datasets (where there was a monotonous decay of the active ester concentration) used for parameter estimation contain the first 10/20 minutes measured values as well.

If the coupled active ester had an initial plateau, zero time of hydrolysis has been assigned to the last point on the plateau before concentration decay.

Parameters whose confidence interval is large and also contains zero are not determined significantly.

Table 3. Measured and estimated concentration of active esters ($[AC]_0$)

Amino acids	$[AC]_0$		conf. interval	
	measured	**estimate**	low	high
Fmoc-Ile-OH	**0.0724**	**0.0684**	**0.0634**	**0.0734**
Fmoc-Val-OH	**0.0950**	**0.0942**	**0.0941**	**0.0958**
Fmoc-Arg(Pbf)-OH	**0.0174**	**0.0238**	**0.0123**	**0.0353**
Fmoc-Ala-OH	**0.0998**	**0.1095**	**0.1030**	**0.1161**
Fmoc-Asn(Trt)-OH	**0.054**	**0.0714**	**0.0595**	**0.0832**
Fmoc-Leu-OH	**0.0688**	**0.069**	**0.066**	**0.071**
Fmoc-GlcAPU(Me)-OH	**0.051**	**0.059**	**0.056**	**0.061**
Fmoc-Thr(^{t}Bu)-OH	**0.059**	**0.059**	**0.0573**	**0.0605**
Fmoc-βAla-OH	**0.132**	**0.136**	**0.1198**	**0.1523**
Fmoc-GalAPU(Me)-OH	**0.0313**	**0.0597**	**0.0564**	**0.0631**
Fmoc-Gly-OH	**0.1061**	**0.1021**	**0.0985**	**0.1058**

ParameterEstimationResults.zip contains three files for each parameter estimation of different active ester hydrolysis kinetics as follows:

***.dat** files contain the input data of the parameter estimation
(Note that some of the data at the and are sometimes ignored.)

***.txt** files contain the output results of parameter estimation
(As produced by COPASI.)

***.tiff** files contain a diagram showing measured data and the fitted curve
(As produced by COPASI.)